George Emílio Sampaio Barreto

ESTUDO DO EFEITO DOS METABÓLITOS DO BENZENO E TOLUENO SOBRE AS MITOCÔNDRIAS CEREBRAIS E HEPÁTICAS DE RATOS

Dissertação apresentada ao Programa de Pós-Graduação em Ciências da Saúde, do Centro de Ciências da Saúde da Universidade Federal do Rio Grande do Norte, como requisito para a obtenção do título de Mestre em Ciências da Saúde.

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Orientador: Prof. Dr. Eryvaldo Sócrates Tabosa do Egito

Co-Orientador: Prof. Dr. Ramon dos Santos El-Bachá

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UNIVERSIDADE FEDERAL DO RIO GRANDE DO NORTE
CENTRO DE CIÊNCIAS DA SAÚDE
PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

Coordenador do Programa de Pós-Graduação em Ciências da Saúde:
Prof. Dr. José Brandão Neto

George Emílio Sampaio Barreto

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Presidente da Banca: Prof. Dr. Eryvaldo Sócrates Tabosa do Egito (UFRN)

BANCA EXAMINADORA

Prof. Dr. Eryvaldo Sócrates Tabosa do Egito (UFRN)

Prof. Dr. Ramon dos Santos El-Bachá (UFBA)

Prof. Dr. Luiz Alberto Lira Soares (UFRN)

Prof. Dra. Lucymara Fassarella Agnez Lima (UFRN)

Aprovada em: 03/11/2005

Dedicatória

Aos meus pais que me deram não apenas o dom da vida, mas ensinamentos que me tornou capaz de enfrentar meus próprios desafios e vencê-los com mérito e poder me orgulhar de mais uma conquista árdua que hoje se transforma em satisfação de mais um projeto de vida concluído.

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ADN – Ácido Desoxirribonucleíco

CYP - CitocromoP450

CYP2E1 - Citocromo P450 da família 2E1

t,t-MA - Ácido trans, trans-mucônico

H_2O_2 - Peróxido de Hidrogênio

$^{\bullet}OH$ - Radical Hidroxil

8-oxodG - 8-hidroxi-2'-deoxiguanosina

APO - Apomorfina

SOD - Superóxido Dismutase

FPG - Formamidopirimidina DNA Glicosliase

EROs - Espécies Reativas de Oxigênio

3MC - 3-Metilcatecol

4MC - 4-Metilcatecol

OC - *o-C*resol

PC - *p-C*resol

MC - *m-C*resol

BZ - Benzaldeído

MB - Metilbenzeno

ADP - Difosfato de Adenosina

ATP - Trifosfato de Adenosina

SNC - Sistema Nervoso Central

L-DOPA - L-3,4-diidroxifenilalanina

Resumo

Objetivo Testar a hipótese de catecóis inibirem a respiração basal associada ao $FADH_2$ em frações mitocondriais hepáticas e cerebrais de rato. Também, procurou-se estudar a ocorrência de danos no DNA induzidos por catecol em células de cérebro de ratos. **Métodos**: Foram avaliados dois catecóis: O pirocatecol (derivado do benzeno) e o 3-metilcatecol (derivado do tolueno); Os homogeneizados de fígado e cérebro de ratos foram incubados com catecol a 1 mM em pH fisiológico. Depois disso, as frações mitocondriais foram isoladas por centrifugação diferencial. O consumo basal de oxigênio foi medido com um eletrodo do tipo Clark após injeção de succinato a 10 mM, e o consumo foi observado por 12 minutos. Em experimentos adicionais, amostras de tecidos cerebrais foram tratadas com pirocatecol em diferentes concentrações (1 mM, 5 mM e 10 mM), durante 0 (sem incubação), 10 e 20 minutos a 37℃, e posteriormente submetidas a eletroforese. **Resultados:** Os resultados demonstram que os catecóis induziram uma inibição parcial da respiração basal mitocondrial associada ao $FADH_2$ de forma dependente do tempo. Já no caso dos experimentos com o ensaio cometa, os dados obtidos demonstram um aumento na ocorrência de cometas de níveis 2 a 4 de forma dose dependente, sugerindo que as espécies reativas de oxigênio geradas pela oxidação do catecol induzem danos de DNA, o que pode também estar relacionado com citotoxicidade deste composto. **Conclusão** O pirocatecol produz inibição da respiração basal associada ao $FADH_2$ em mitocôndrias isoladas de fígado e cérebro de ratos; o 3-metilcatecol, nessa mesma concentração, produziu toxicidade semelhante no modelo mitocondrial. Adicionalmente, o pirocatecol induziu a um dano de DNA em células cerebrais de ratos, observado na formação de cometas e conseqüente degradação do DNA.

Palavras-Chave: benzeno, tolueno, catecóis, radicais livres, respiração.

1 INTRODUÇÃO

Benzeno e tolueno são compostos clastogênicos, carcinogênicos e genotóxicos, que causam uma histórica preocupação no tocante a um potencial risco à saúde ocupacional. Redução progressiva e contínua no uso do benzeno, dentro dos limites de exposição ocupacional, tem assegurado que os efeitos decorrentes das altas concentrações do benzeno no ambiente de trabalho devem representar um problema sério, exceto nos casos de acidente. Presente preocupação é centrada nos efeitos a uma exposição continua em longo prazo de baixas concentrações de benzeno[1].

Baseado em estudos epidemiológicos, a exposição humana ao benzeno causa depressão da medula óssea, mielotoxicidade, câncer linfohematopoiético, particularmente leucemia não-linfocítica aguda e câncer nasofaríngeo. Um estudo realizado por Liu et al. [2] encontrou que as concentrações de benzeno entre 40-200mg/m^3 e >200mg/ m^3 resultaram em um significante aumento das concentrações do marcador de DNA oxidado, 8- hidroxideoxiguanosina (8-oxodG), em trabalhadores de uma fábrica de sapatos comparado com um grupo controle. Muitas destas evidências derivam-se de estudos industriais de trabalhadores expostos ao benzeno, geralmente como um constituinte de uma mistura complexa. Estes incluem a fabricação de calçados, petroquímica, química e indústrias manufatureiras de borracha [3-10].

O fígado é o local de metabolização do benzeno e o principal sistema enzimático metabólico é o citocromo P450 (primariamente CYP2E1). Este processo

envolve a formação de uma série de metabólitos reativos tais como fenol, hidroquinona e catecol (1,2-diidroxibenzeno).

Catecóis são os produtos intermediários da degradação de compostos aromáticos. Em humanos e outros mamíferos, podem ocorrer como metabólitos da degradação do benzeno e estrógenos [11]. Por sua vez, microorganismos e determinados tipos de bactérias podem produzir catecóis através do seu processo metabólico.

Catecol se auto-oxida com facilidade, levando à geração de semiquinonas e quinonas reativas, e inevitavelmente, à formação de espécies reativas de oxigênio (EROs). Estes radicais livres podem ser tóxicos para células e organismos se não forem eliminados. EROs podem agir como pro-oxidantes, danificando macromoléculas, tais como DNA e proteínas e destruindo o funcionamento de membranas, possivelmente devido à sua atividade cíclica redox [12-15]. Nosso laboratório recentemente reportou que a citotoxicidade induzida pelos catecóis em culturas de células gliomatosas (GL-15) é devido à produção de superóxido e quinonas reativas [16].

2 REVISÃO BIBLIOGRÁFICA

2.1 Benzeno: Toxicidade e Metabolismo

Benzeno é uma substância usada principalmente como um precursor da síntese de numerosos produtos incluindo drogas, tinturas, inseticidas, plásticos, e está presente na gasolina, fumaça de cigarros e emissões industriais, sendo por isso facilmente encontrada no meio ambiente enquanto poluente [17-19]. Baseado em estudos epidemiológicos, a exposição humana ao benzeno causa depressão da medula óssea, leucemia mielóide aguda, leucemia linfocítica aguda, linfoma de Hodgkin e cânceres do pulmão e nasofaríngeo [20-21]. O efeito agudo do benzeno em altas doses reflete sua atividade como um anestésico geral e pode levar a uma depressão do sistema nervoso central, perda da consciência e uma sensibilização do miocárdio às catecolaminas. A exposição crônica pode resultar em depressão da medula óssea, expressa em uma leucopenia, anêmica e trombocitopenia, levando a uma pancitopenia e anemia aplástica [22].

É geralmente aceito que para o benzeno exercer sua toxicidade, este precisa ser primeiro metabolizado no organismo pelos citocromos P450 (primariamente CYP2E1), resultando em fenol, catecol **(Figura 1)** e hidroquinona [23] .

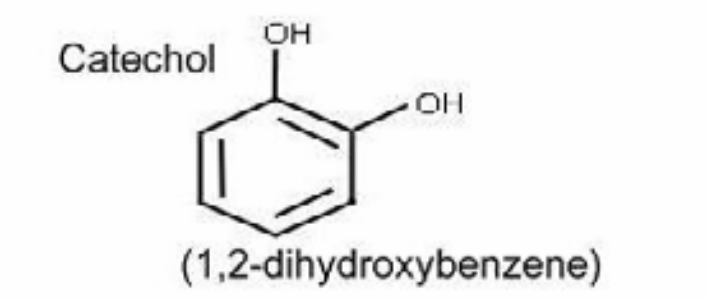

Figura 1: Fórmula Estrutural do catecol (http://www.thejimkelly.com/freds-garden)

O benzeno é primeiramente hidroxilado no fígado pela atividade do CYP2E1 a fenol. Quando a atividade da CYP2E1 resulta na formação do óxido de benzeno, ele pode ser rearranjado não-enzimaticamente para formar o fenol, ou pode levar à formação do 1,2-benzenodiol (1,2-BD, catecol). O fenol pode ser posteriormente hidroxilado para formar catechol ou 1,4-benzenodiol (1,4-BD, hidroquinona). Ambos podem ser, por conseguinte, hidroxilados a 1,2,4-benzenotriol (1,2,4-BT). Adicionalmente, 1,4-BD pode ser oxidado a *p*-benzoquinona na qual pode ser reduzido a 1,2,4-benzenotriol seguindo uma redução de 2 elétrons catalisadas pela DT-diaforase [20]. O metabólito final, 1,2,4-benzenotriol, mesmo formado em pequenas quantidades, é capaz de potentes efeitos tóxicos. Este metabólito é conhecido por induzir troca de cromátides-irmãs, aberração cromossômica em culturas de linfócitos humanos [24-25]. Adicionalmente, espécies reativas de oxigênio produzidas através da auto-oxidação do 1,2,4-benzenotriol pode causar quebras do DNA, danos em microtúbulos, formação de micronúcleos e aneuploidia do cromossomo 9 em células HL-60 [26-28]. Muitos metabólitos do benzeno, incluindo o ácido S-fenilmercaptúrico, t,t-MA (ácido trans,trans-mucônico), hidroquinona, fenol e catecol têm sido utilizados como marcadores biológicos em humanos expostos ao benzeno [29-31]. Estes metabólitos podem acumular na medula óssea, onde podem ser

bioativados pelas mieloperoxidases e outras peroxidases, por exemplo, de heme-proteínas, para dar origem a quinonas e semiquinonas reativas e à formação de espécies reativas de oxigênio [32]. **(Figura 2)**.

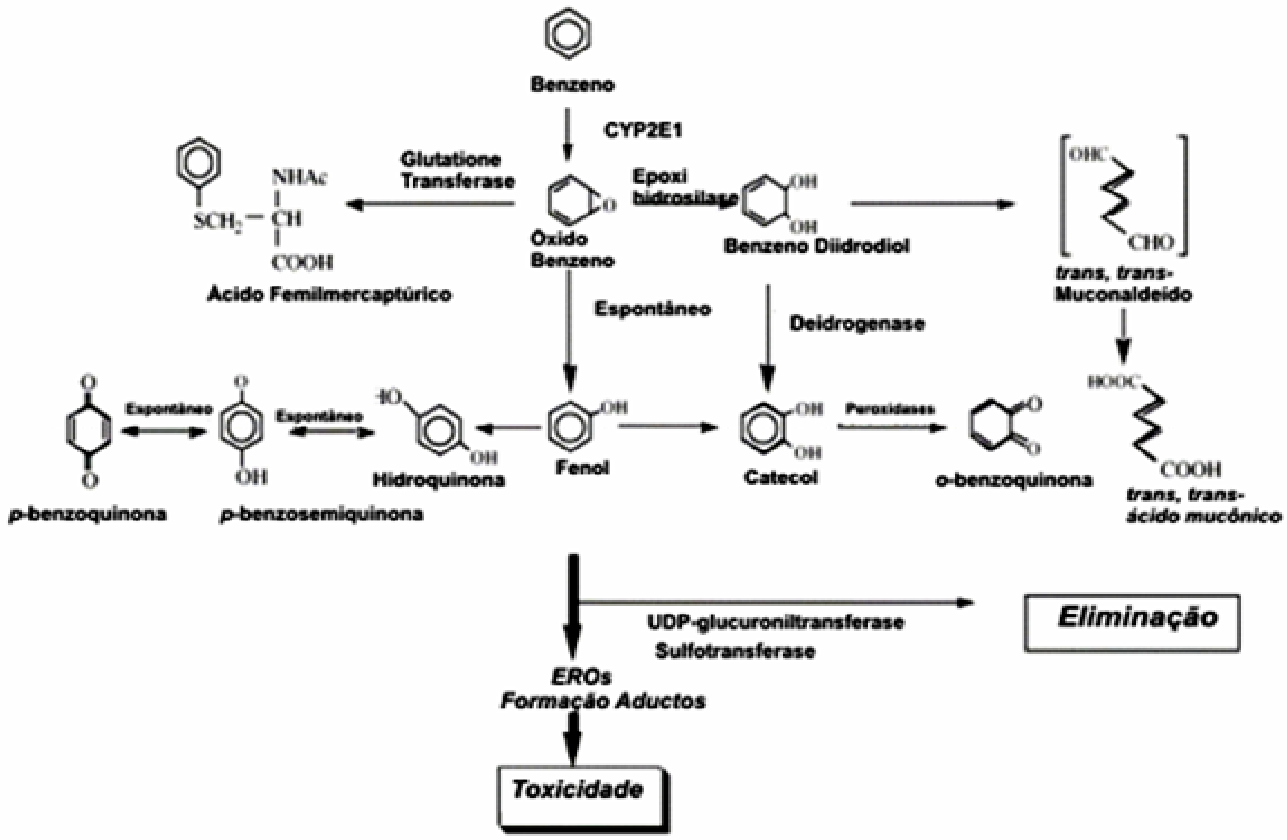

Figura 2: Cascata da Bioativação do Benzeno. Benzeno pode ser bioativado pelos citocromos P450 2E1 (CYP2E1) a óxido de benzeno. Este pode ser transformado em fenol, reagindo com glutatione para formar ácido fenilmercaptúrico, ou ser hidroxilado para formar um dihidrodiol. O benzeno dihidrodiol pode levar à produção de ácido mucônico e catecol. Fenol pode também levar à formação de catecol e hidroquinona, ambos das quais podem gerar quinonas e semiquinonas reativas. Se não eliminados, estes metabólitos podem produzir espécies reativas de oxigênio, no qual podem danificar alvos macromoleculares essenciais incluindo DNA, proteínas e lipídios (Baseado em Schweigert, 2001).

2.2 Tolueno: Um composto volátil

A inalação involuntária ou deliberada de solventes tem sido largamente reconhecida devido aos seus efeitos tóxicos e mudanças comportamentais que provoca. Várias organizações governamentais relacionadas à saúde humana têm reportado que o uso indiscriminado de solventes industriais tem crescido vertiginosamente [33]. Altas concentrações de solventes hidrocarbonados são capazes de induzir a um agudo estado reversível de narcose, mas tem sido difícil relatar alterações histológicas crônicas irreversíveis no sistema nervoso central. Tem sido reportado que a exposição prolongada a baixos níveis de solventes leva a mudanças comportamentais e fisiológicas em determinadas populações de trabalhadores [34-36]. A inalação crônica de tolueno puro leva a disfunções cerebelares irreversíveis e do trato piramidal, e a inalação crônica de tolueno sob a forma de adesivos pode causar uma psicose paranóica irreversível, uma alta incidência de epilepsia do lobo temporal e a uma diminuição do QI [37]. Hidrocarbonetos aromáticos, tais como o tolueno, em altas concentrações, têm demonstrado causar um aumento dos radicais livres e EROs no sistema nervoso central de mamíferos [38-40]. Estudos demonstram uma perda de 16% de neurônios na região inferior (CA3 and CA2) de ratos expostos a 1500 ppm de tolueno por um período de seis meses. O tolueno pode afetar a atividade elétrica no cérebro e neurotransmissores e sua inalação diminui os níveis de noradrenalina (NA) na região dorsal de ratos, uma área contendo o locus ceruleus onde as células noradrenérgicas estão localizadas [41]. Estudos demonstraram, adicionalmente, que tal inalação diminui os níveis de dopamina (DA) no hipotálamo e na parte médio-ventral do cérebro, contendo a substância negra na qual as células dopaminérgicas estão centradas.

O tolueno ocorre naturalmente em óleo bruto (constituindo cerca de 80% da combinação de solventes) e é comumente encontrado como um contaminante na superfície como um resultado das atividades de estoque e depósito de lixo. Transformação biológica e volatilização são os principais mecanismos de remoção do tolueno em solos e água contaminados. O fígado é o principal local de metabolização do benzeno, tolueno, etilbenzeno e xileno (BTEX), sendo o P450 2E1 o mais eficiente sistema enzimático que oxida BTEX a metabólitos solúveis em ratos e microssomos hepáticos humanos [42]. Citocromo P450 2B1 e 2E1 em ratos são essenciais para a ativação do metabolismo dos derivados do BTEX a potenciais produtos genotóxicos [43].

O tolueno é biodegradado por uma variedade de microorganismos que usam o mesmo como uma fonte de carbono. O tolueno pode ser aerobicamente transformado via cinco processos, com uma inicial hidroxilação para posições *orto* [44], *meta* [45], ou *para* [46], ou no grupo metil [47], ou com uma deoxigenação nas posições 2,3 [48]. Os principais produtos da biotransformação incluem o 4-metilcatecol (4MC), catecol (CAT), *o*-cresol (OC), *p*-cresol (PC), *m*-cresol (MC), benzaldeídeo (BZ), e metilbenzoato (MB) e 3-metilcatecol (3MC) – **Figura 3**.

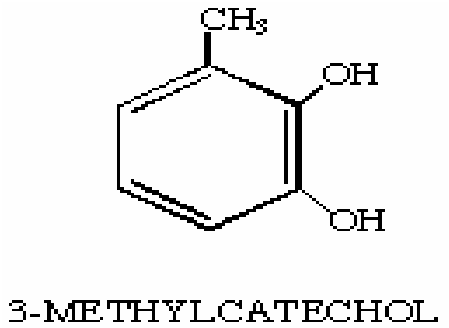

Figura 3: Fórmula Estrutural do 3-metilcatecol
(http://www.chem.umn.edu/class/3306/old/spring9596/presentation/toluene/comp/tol_compIX.html)

2.3 – Catecóis: Efeitos Tóxicos em Macromoléculas

Catecóis são compostos que possuem 2 posições vicinais na posição orto, e um anel benzênico em sua composição; são encontrados na natureza como biomoléculas essenciais tipo dopa, dopamina, adrenalina e noradrenalina. Existe uma relação positiva entre a lipofilicidade e o coeficiente de permeabilidade da barreira hematoencefálica [49], o que facilita o acesso de substâncias aromáticas ao sistema nervoso central (SNC). A toxicidade dose-dependente de catecóis endógenos como L-3,4-diidroxifenilalanina (l-DOPA), dopamina e também outras drogas que possuem um grupamento catecólico, como apomorfina, está bem documentada em estudos *in vitro* [50-52]. Há um crescente interesse em se estudar a toxicidade de catecóis endógenos em modelos celulares específicos desde que tem sido especulado que essa toxicidade está associada a doenças neurodegenerativas. Disfunções mitocondriais cerebrais e estresse oxidativo estão associados à fisiopatologia da Doença de Parkinson. Estudos recentes de Betarbet e col. [53] mostram que o tratamento crônico de ratos com rotenona, inibidor mitocondrial do complexo I, resulta em degeneração seletiva dopaminérgica nigroestriatal, incluindo formação de corpos de Lewy e perda celular. Porém, a concentração de rotenona administrada foi insuficiente para inibir a respiração mitocondrial, indicando que um defeito bioenergético com a depleção de ATP não poderia explicar a neurodegeneração pronunciada. Sob essas condições, um aumento da produção mitocondrial de espécies reativas de oxigênio, secundária a uma inibição parcial do complexo I, poderia contribuir para uma degeneração dopaminérgica nigroestriatal induzida pela rotenona [54-56]. Estes dados revelam que mediante uma privação quer seja parcial ou total de oxigênio, a fosforilação oxidativa pode estar comprometida e,

consequentemente, haverá uma maior formação incompleta de oxigênio reduzido e por fim, a geração de EROs.

2.3.1 – Espécies Reativas de Oxigênio

Os alvos moleculares susceptíveis aos danos causados pelas EROs incluem proteínas, lipídios e o DNA, sendo que alguns processos patológicos podem advir desse fenômeno como, por exemplo, alguns tipos de tumores. Para suportar essa idéia, estudos realizados por Kolachana e col [57] e Shen e col [58], demonstraram que os metabólitos do benzeno iniciam um processo oxidativo com conseqüente dano em células HL60 e, segundo Gaido e Wierda [59], tais metabólitos causam oxidação lipídica em modelos animais, enfatizando ainda mais o papel de EROs na toxicidade causada pelo benzeno.

EROs como o ânion superóxido, radical hidroperoxil, peróxido de hidrogênio e radicais hidroxil altamente reativos são gerados em muitos processos fisiológicos. Enquanto a célula tem desenvolvido mecanismos não-enzimáticos e enzimáticos para eliminar EROs, o estresse oxidativo pode ocorrer como uma ativação xenobiótica, levando a um desequilíbrio entre a formação de EROs e a desintoxicação celular, favorecendo a geração de espécies reativas [60].

A excessiva geração de EROs é presumida ser um fator significante na injúria tecidual observada em muitos estágios de diversas patologias. Existem inúmeros locais de produção de EROs dentro das células, incluindo enzimas da família dos citocromos P450, xantina oxidase e a cadeia transportadora mitocondrial de elétrons.

A cadeia transportadora de elétrons é uma permanente fonte de estresse oxidativo. Cerca de 1 a 3% do oxigênio consumido é incompletamente reduzido e leva à produção de EROs [61]. Tem sido constatado que durante a Fase 4 da respiração mitocondrial há a produção de superóxido e peróxido de hidrogênio a níveis que constituem cerca de 2% do total do consumo mitocondrial de oxigênio [62]. Indicações de estresse oxidativo incluem depleção de glutation reduzido, acúmulo de ferro e a presença de produtos oxidativos lipídicos, protéicos e de DNA [63-65]. Excessiva produção de EROs na célula pode ser induzida por um número de xenobióticos, íons de metais de transição e radiação ultravioleta e ionizante. Adicionalmente, sob condições da produção aumentada de EROs, ou inabilidade do sistema de defesa antioxidante, EROs pode acumular, exercendo um potente efeito danoso nas células e por fim em todo o organismo [66-71]. A mitocôndria, sendo o principal local de geração de EROs na célula, é também seu principal alvo. Este efeito, em contrapartida, resulta em dano à cadeia respiratória mitocondrial e, como conseqüência, um aumento adicional da produção de radicais livres. Um ciclo vicioso é então formado [72], que pode ser considerado um agente causador de um grande número de disfunções mitocondriais associadas à idade e também um dos mecanismos da morte celular programada (apoptose). A disfunção mitocondrial é particularmente identificada por uma inibição parcial (20-40%) da atividade do complexo I da cadeia respiratória [73-74], presente também em tecidos periféricos [75-76]. Após inibição mitocondrial, substratos glicolíticos previnem a morte celular promovendo uma fonte alternativa de ATP celular. ATP-ases são estimuladas a hidrolisarem o ATP formado pela glicólise e, com isso, a proteção é perdida. Os componentes da transferência de elétrons anormalmente reduzidos pela inibição da respiração, especialmente a ubiquinona, reagem diretamente com o oxigênio para

formar radicais tóxicos. Mitocôndrias também geram oxigênio reativo após exposição a químicos oxidantes. A conseqüência é uma desregulação do mecanismo de transição da permeabilidade mitocondrial, o que leva a uma depleção do ATP e perda da viabilidade celular. Por fim, mitocôndrias são tanto uma fonte quanto um alvo de radicais tóxicos de oxigênio na injúria celular [77].

2.3.2 – EROs e dano de DNA

Catecóis são genotóxicos bem conhecidos. O fato de poderem se ligar covalentemente a moléculas celulares, proteínas e DNA, em tecidos está implicada no mecanismo de toxicidade (inibindo a replicação celular) e carcinogenicidade (iniciação da leucemia [20]). É também pressuposto que ajam como mutágenos via um mecanismo indireto, levando a um dano oxidativo do DNA através da formação de radicais hidroxil via peróxido de hidrogênio [78].

Catecóis podem ser transformados, após metabolização *in vitro*, a benzoquinonas, que são compostos potencialmente hematotóxicos, genotóxicos e carcinogênicos [79-80]. A exposição de células de embrião de hamster sírio ao catecol induz a uma transformação celular, mutação gênica, desordenada síntese de DNA, aberrações cromossômicas e intercâmbio de cromátides-irmãs [81]. Catecóis induziram à formação de micronúcleos em linfócitos da medula óssea de ratos *in vivo* [82-83]. *In vitro*, estudos conduzidos em linfócitos humanos demonstraram, que o catecol significantemente induziu à formação de micronúcleos e também aumentou o número células micronucleadas [84]. O estudo de misturas binárias e ternárias desses compostos mostrou a existência de interações sinérgicas e antagônicas com

respeito à indução de micronúcleos em linfócitos humanos e em outras linhagens celulares humanas e de hamsters [83,85]. O mecanismo molecular da mutagenicidade de compostos fenólicos em células de mamíferos não está bem estabelecido. Alguns compostos fenólicos tais como o catecol, são conhecidos pela sua propriedade de auto-oxidação em soluções aquosas em um pH fisiológico, levando à formação de espécies reativas de oxigênio, tais como ânion superóxido [86]. Estas insertivas sugerem que os mecanismos de indução ao dano do DNA por estes compostos poderiam ser mediados pela produção de espécies radicais livres.

Com o pressuposto de que a formação de EROs, nesse contexto, possa advir de uma disfunção mitocondrial, este trabalho teve o intuito de estudar, de uma maneira mais aprimorada, os efeitos de catecóis em um modelo mitocondrial, procurando evidenciar e esclarecer os mecanismos de toxicidade desses compostos aromáticos. Tendo em vista o vasto estudo acima descrito que descreveu o grande efeito tóxico induzido por catecóis, o objetivo deste estudo foi investigar a atividade de catecóis em frações mitocôndriais de fígado e cérebro de ratos para testar a hipótese desses compostos agirem como inibidores da respiração basal. Paralelamente, testamos a hipótese dessas moléculas serem indutores de danos ao DNA em células cerebrais de ratos.

2.4 Objetivos Específicos

2.4.1 Realizar uma ampla revisão para discutir e elucidar os efeitos desencadeados pelos catecóis em diversos sistemas orgânicos (com ênfase aos estudos *in vitro* e *in vivo*).

2.4.2 Estudar o efeito de catecóis em mitocôndrias cerebrais e hepáticas e sua relação com a fosforilação oxidativa e consumo basal de oxigênio.

2.4.3 Analisar o comportamento de catecóis a nível celular, procurando evidenciar prováveis danos mitocondriais e ao DNA celular.

Inicialmente foi feita uma revisão sobre a propriedade do catecol de induzir a um dano de DNA. Em seguida, foi analisada a capacidade desse composto de levar a uma inibição parcial da respiração mitocondrial hepática ligada ao $FADH_2$. No terceiro momento foi investigada a inibição da respiração mitocondrial cerebral de ratos, expostos ao 3-metilcatecol. Como conseqüência dos resultados encontrados nestes dois estudos, avaliamos a capacidade do catecol causar dano ao DNA em células cerebrais de ratos. E, finalmente, seguindo a mesma metodologia dos estudos feitos com o 3-metilcatecol, avaliamos a hipótese do pirocatecol induzir a uma disfunção da respiração basal mitocondrial em cérebro de ratos.

3 ANEXAÇÃO DE ARTIGOS

3.1 Artigo I – A review about the role of catechol, a benzene metabolite, on DNA damaging

Na literatura consultada existe um grande número de trabalhos de investigação que avaliam, tanto *in vitro* e *in vivo* quanto em estudos clínicos, os efeitos da exposição prolongada a baixos níveis de benzeno. No entanto, a atual preocupação tem sido estabelecida com respeito a trabalhadores expostos por um curto período.

Contudo, o composto largamente utilizado nesses estudos é o benzeno, o que deixa a desejar, já que para o benzeno exercer sua toxicidade é necessário ser primeiramente metabolizado. Então, entende-se que a citotoxicidade é devido à sua biotranformação a catecóis.

Apesar de muitos estudos serem conflitantes e certamente não fornecerem informações suficientes para um entendimento coerente sobre os efeitos dos catecóis, derivados do benzeno, em diversos sistemas orgânicos, este trabalho teve o intuito de idealizar um pensamento lógico e criar um raciocínio crítico sobre os prováveis efeitos desses compostos aromáticos.

O abstract deste artigo foi aceito pelos editores do periódico **Current Pharmaceutical Design**, conforme documento, em anexo, na página 15.

A cópia do artigo, o qual está em fase final de revisão, encontra-se nas próximas páginas (16 a 51).

De: KAZIM [mailto:kazimits@cyber.net.pk]
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REVIEW

A REVIEW ABOUT THE ROLE OF CATECHOL, A BENZENE METABOLITE, ON DNA DAMAGING

George Emílio Sampaio Barreto[1], Leonam Gomes Coutinho[2], Ramon dos Santos El-Bachá[3], Lucymara Fassarella Agnez Lima[2] and Eryvaldo Sócrates Tabosa do Egito*

[1] M. Sc. student of the Graduation Program in Health Sciences (PPGCSA), Health Sciences Centre. Laboratory of Dispersed Systems (**LASID),** Department of Pharmacy, Federal University of Rio Grande do Norte (UFRN), Natal – RN - Brazil.

[2] Laboratory of Molecular Biology and Genomics. Department of Celular Biology and Genetics. Federal University of Rio Grande do Norte (UFRN), Natal – RN.

[3] Ph. D., Head of Laboratory of Neurochemistry and Cell Biology (LabNq), Department of Biochemistry and Biophysics, ICS, Federal University of Bahia (UFBA), Salvador – BA – Brazil.

* **Corresponding author:**
Professor E. S. T. Egito, Ph. D.
Head of **LASID,** Department of Pharmacy, UFRN
Rua Praia de Areia Branca, Natal - RN – Brazil (socrates@ufrnet.br).

Abstract

Benzene is a widespread volatile compound. Long-term exposure to this substance has been extensively studied, because of its important toxic effects in workers exposed to low levels. Alterations such as leukemia, blood disorders, bone marrow depression and some types of cancer are strictly related to benzene-initiated toxicity. Bioactivation of benzene can lead to the formation of hazardous metabolites such as phenol, hydroquinone and catechol. Therefore, the latter one can be oxidized to form semiquinones and reactive quinones which are presumible to play an important role in reactive oxygen species (ROS) generation. ROS formation can directly induce DNA single and double-strand breaks, oxidized nucleotides, hyper-recombination and consequently deleterious genetic changes. In order to evaluate DNA damage, alkaline single-cell gel electrophoresis (Comet Assay) has been widely used, and has shown to be a very sensitive method to detect genetic damage. In this review, it has been assigned the cytotoxic effects of benzene and its main metabolite, catechol, towards macromolecules and a potential role for DNA damaging. Such information can be quite important for further pharmaceutical design of new drug carries for gene therapy (A review with 165 references).

Key Words: benzene; catechol; toxicity; reactive oxygen species; DNA; comet assay

1- INTRODUCTION

Benzene is an aromatic hydrocarbon substance that has cytotoxic, haematotoxic, immunotoxic and genotoxic properties [1-16], and is extensively used in industry as a volatile solvent or an assorting material for the synthesis of other chemicals [17]. Currently, concern is focused on the effects of long-term occupational and environmental exposure to low levels of benzene [18]. Since the latter has been reduced, and in some workplaces has been supplanted by other solvents, industrialization inevitably leads to more benzene in the environment [19].

Exposure to benzene is strictly related to some industrial activities (e.g. coating applications, rubber, chemical, and shoe production). Furthermore, the presence of this xenobiotic in cigarette smoke and gasoline (i.e. especially after the reduction of lead content and the consequent decrease in octane number) renders benzene exposure an environmental as well as an occupational problem.

1.1- Benzene: Toxicity and Metabolic Pathway

Benzene is an industrial and environmental contaminant that continues to be a significant medical concern. In humans, acute myelogenous leukemia is the major health risk associated with exposure to low levels of benzene [20-21]. Nevertheless, the mechanism by which benzene exerts its long-term toxic effects, such as causing acute myeloid leukaemia and myelodysplastic sydromes in exposed workers, remains largely unclear [22-23].

Benzene to exert its toxicity must be first metabolized in the liver by the activity of Cytochrome P450 (CYP). However, none of the proeminent metabolites of benzene are hard

electrophiles. Benzene is first hydroxylated in the liver by the activity of CYP2E1 to yield phenol [24]. When the activity of CYP2E1 results in the formation of benzene oxide, it can rearrange non-enzimatically to form phenol, or can lead to the formation of 1,2-benzenediol (1,2-BD, catechol). Phenol can be further hydroxylated to form catechol or 1,4-benzenediol (1,4-BD, hydroquinone), both of which can be further hydroxylated to 1,2,4-benzenetriol (1,2,4-BT). Moreover, 1,4-BD may be oxidized to *p*-benzoquinone which can be reduced to 1,2,4-benzenetriol following a two-electron reduction catalyzed by DT-diaphorase [21]. The ultimate metabolite, 1,2,4-benzenetriol, although formed in small quantities, is capable of potent toxic effects. This toxic metabolite is known to induce sister-chromatid exchanges, micronuclei and chromosomal aberration in cultured human lymphocytes [25-26]. Moreover, active oxygen species produced through auto-oxidation of 1,2,4-benzenetriol can cause strand breaks, microtubule damage, micronuclei and aneuploidy of chromosome 9 in HL-60 cells [27-29]. Many benzene metabolites, including S-phenylmercapturic acid, (t, t-MA; trans, trans-muconic acid), hydroquinone, phenol and catechol have been utilized as biomarkers in humans exposed to benzene [30-32].

Fig. (1)

Numerous reviews over the years have described and evaluated the adverse health effects associated with benzene exposure [33]. This volatile compound has been established to be a carcinogen of note, and results in panoply of blood disorders, most notably myelodisplastic syndrome, and acute and chronic myelocytic leukemia, in workers in various industries. Therefore, the major concern regarding this solvent centers on the effects of long-term exposure, both occupationally and environmentally, to low concentrations of benzene [30-33].

Benzene is known to cause chromosomal aberrations in vitro. Available data suggest that prolonged exposure to benzene at 64mg/m^3 may be associated with chromosomal aberrations [33]. Other reported genetic aberrations attributed to benzene involve sister chromatid exchanges, DNA cross-linking, DNA adducts formation [34]. It has also been reported that benzene causes higher levels of DNA single strand cleavage in B-lymphocytes then in T-lymphocytes and granulocytes of workers exposed to this environmental pollutant [35]. According to the results of Sul et al. [36], DNA damage in lymphocytes exhibited a strong correlation with the breath benzene and urinary t, t-MA levels, but not with phenol levels. It was also reported that DNA damage in individual T and B-lymphocytes is clearly associated with t, t-MA levels in workers exposed to low levels of benzene at the low level in a company [35].

Hematotoxicity caused by benzene exposure is thought to occur via the metabolites phenol, catechol and hydroquinone that can be further metabolized to reactive intermediates including benzoquinone. Win [37] showed that phenol, hydroquinone, benzoquinone and catechol, all metabolites of benzene, caused a dose-dependent increase in frequency of homologous DNA recombination in the CHO 3-6 recombination cell line. Previous studies have shown that exposure to benzene can increase DNA recombination in other mammalian cell lines but at concentrations that were 3-fold higher than those used in this experiment [38-39]. This increased frequency of recombination can be completely blocked by the activity of the antioxidative enzyme catalase, supporting the hypothesis that increased oxidative stress plays a role in benzene-initiated toxicity. Thus, Winn [37] demonstrated that benzoquinone, a metabolite of benzene, is the most potent metabolite in its ability to increase recombination. Similar results were obtained by Sze et al. [40], who found that benzoquinone, of all the

benzene metabolites that they tested, showed the highest potency in inducing DNA strand breaks in CHO cells.

Benzene metabolites such as catechol bind covalently to proteins and DNA in biological systems such as cells or tissues, thereby inducing intracellular toxic effects, such as the inhibition of cell replication or carcionogenesis [17]. Although many studies on chromosome aberrations [9, 41-43], sister chromatid exchanges [9, 41, 43-44] and micronuclei [45] have shown that benzene exposure induced DNA damage, unequivocal positive results are rarely found in human biomonitoring study with these classical cytological tests [46].

The enzymatic bioactivation of benzene leading to the formation of ROS and subsequent increased oxidative stress is thought to play a significant role in benzene-initiated toxicity. Mice treated with benzene, phenol, catechol and hydroquinone have significantly increased levels of oxidized DNA [47]. Furthermore, bone marrow cells from benzene-treated mice have increased DNA binding activity for the transcription factor activator protein-1 (AP-1), a known target of oxidative stress [48]. These findings are consistent with increased levels of ROS after benzene exposure. Benzene metabolites have also been shown to increase myeloid cell growth in vitro by the formation of ROS [49]. Results from Winn [37] demonstrated that the antioxidative enzyme catalase can completely block the observed increase in homologous DNA recombination initiated by exposure to phenol, catechol, hydroquinone, or benzoquinone, supporting the hypothesis that ROS can mediate the toxicity observed with exposure to these metabolites. Furthermore, these results are consistent with studies demonstrating that exposure to carcinogens including benzene leads to increased DNA recombination in the yeast *Saccharomyces cerevisiae* [50-51], which can be reduced by the presence of the free-radical scavenger N-acetyl cysteine [51]. The protective effects of

catalase observed by Winn [37] are consistent with numerous in vitro studies showing a protective effect of catalase against ROS production and ROS-initiated damage [52-54].

1.2- Catechols: Toxic actions towards macromolecules

Catechols readily undergo auto-oxidation in an aqueous solution, under a physiological pH, to form semiquinones radicals and quinones, which are more reactive than catechols. The mechanisms most frequently cited to explain the toxicity of catechols are: (i) the generation of reactive oxygen species by redox reactions; (ii) DNA damage in the form of oxidative damage or DNA arylation; (iii) protein damage by sulfhydryl arylation or oxidation; and (iv) interference with electron transport in energy transducing membranes [55-56]. Moreover, catechol releases iron from ferritin inducing lipid peroxidation in brain homogenates [57]. When catechol is oxidized enzimatically or in the presence of oxygen and heavy metals, one electron is transferred to the molecular oxygen, and consequently superoxide (O_2^-) is formed. In the presence of heavy metals (e.g. cooper, iron), superoxide is further reduced to hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$). Catechol can act as pro-oxidant damaging macromolecules such as DNA and proteins and destroying membrane functioning due to their redox cycling activity [56]. Pereira et al. [58] reported that catechol-induced cytotoxicity towards glioblastoma cells *in vitro* is due to the production of superoxide and reactive quinones.

Although the exact mechanism for benzene-induced toxicity is unknown, it seems that catechol plays an important role regarding the effects of the exposure to this molecule. The lethal human dose of catechol is 450-4540 nmol/kg or one teaspoon given once for a 70 kg person. Therefore, Barreto et al. [59] investigated the toxic effect of 1mM catechol towards

liver subcellular fracton since this organ is responsible for xenobiotic metabolism. This concentration is in the range of the catechol level found accumulated in people exposed to action of this compound (90-9000 µM) [60].

Catechols are not only a chemical but are also present in certain foods, such as anions, crude beet sugar, coffee and somked fish. It has been shown to be carcinogenic in rodents. In several experiments in rats and mice involving administration with known carcinogens, catechol strongly enhanced incidence of papillomas of the tongue, carcinomas of the oesophagus, squamous cell carcinomas of the forestomach and adenocarcinomas [61-67]. Catechol is a major metabolite of benzene, which is known to cause leukemia in humans and animals [68-71]. Since the International Agency for Research on Cancer (IARC) has classified catechol as a group 2B carcinogen [72], which is possible carcinogenic to human. However, the mechanism of DNA damage to elicit its carcinogenicity catechol has not been clarified.

Catechol as such does not cause oxidative DNA damage in vitro. However, combined with heavy metals (e.g. Cu2+, Fe3+) and in the presence of molecular oxygen, DNA strand breaks can be observed [73-74]. DNA strand breaks are caused by a redox reaction of Cu (II) and catechol to yield Cu (I) and the semiquinone radical and a subsequent cooper catalysed reduction of molecular oxygen, where superoxide and hydrogen peroxide are formed. A DNA-cooper-oxo complex [DNA-Cu(I)-OOH] finally causes the DNA strand breaks by splitting of hydroxyl radicals in the vicinity of the DNA [75]. This is a possible mechanism of oxidative DNA damage by catechol and NADH in the presence of Cu^{+2} . It is reasonable to speculate that catechol undergoes Cu^{+2} – mediated autoxidation to generate Cu^{+} and semiquinone radical. Cu^{+} reacts with O_2 to generate O_2^{-} and subsequently H_2O_2. Formed Cu^{+}

binds to DNA with H_2O_2, resulting in the formation of a Cu (I)-hydroperoxo complex such as DNA-Cu(I)OOH [76]. Furthermore, the reactive oxygen species can be produced abundantly through the reduction of the oxidized form, such as semiquinone radical or 1,2-benzoquinone, by NADH non-enzimatically. The Cu (I)-hydroperoxo complex may be considered to be a bound hydroxyl radical, which can release •OH causing DNA damage. The $^{\bullet}OH$ released from a bound hydroxyl radical immediately attacks an adjacent constituint of DNA before it can be scavenged by $OH^{\bullet}$ scavengers [77]. In addition, catechol-type compounds, such as carcinogenic catechol estrogens and flavonoids, also induced oxidative DNA damage through H_2O_2 generation [78-80]. The binding of cooper to DNA and/or protein in chromatin is proposed to serve physiological functions [81], whereas cooper bound to DNA and/or protein may provide an adventious site for deleterious redox reactions [82]. Cooper ions bind to non-histone proteins and cause much stronger ascorbate-mediated DNA damage than iron [83]. Several studies have indicated that cooper has the ability to catalyze the production of reactive oxygen species and to mediate oxidative DNA damage [77, 84-87].

Fig. (2)

In vivo, DNA damage experiments have been performed with bacteria and mammals. A peroxide evolution, indicating a redox cycling of catechol, was measured in *Escherichia coli* cells, exposed to catechol or combination of catechol and copper [74]. Despite of the peroxide production, catechol is not mutagenic in *E.coli and Salmonella typhimurium*, when tested alone or in combination with cooper [74, 88]. The absence of mutagenicity is probably due to activated defence systems as for example an induction of a catalase. Catalases reduce hydrogen peroxide to water and molecular oxygen and detoxify organic peroxides [74]. Therefore, no DNA-cooper-oxo complexes are formed and no additional DNA damage occurs. Catechol alone does not cause oxidative DNA damage in bone marrow as shown by

Kolachana et al. [47]. In combination with phenol or hydroquinone (neither compounds induce oxidative DNA damage alone), however, the 8-hydroxy-2´-deoxyguanisine (8-oxodG) level increases, which is indicative of oxidative DNA damage [47]. Catechol exhibits inhibitory effects on the DNA synthesis in the mouse lymphoma cell line L5178YS. These effects have been explained by DNA damage caused by DNA alkylation or oxidative DNA damage [47]. But similar effects in human T lymphoblasts have been explained by enzyme inhibition.

Oikawa et al. [89] demonstrated that the content of 8-oxodG in HL-60 cells was increased by catechol, whereas the content of 8-oxodG in HP100 cells was not increased. DNA base damage in the form of 8-oxodG is a prominent indicator of oxidative stress and has been well-characterized as a premutagenic lesion in mammalian cells [90-92]. Catalase activity of HP100 cells was 18-fold higher than that of HL-60 cells [93]. These results suggest that catechol is capable of causing oxidative DNA damage in human cultured cells, and that generation of H_2O_2 plays a critical role in catechol-mediated DNA damage. Numerous studies have indicated that the formation of 8-oxodG causes misreplication of DNA that may lead to mutation or cancer [92-94]. Thus, oxidative DNA damage seems to be relevant to the carcinogenic process of catechol.

Oikawa et al. [89] showed that DNA ladder formation, which is associated with apoptosis, by 50 μM catechol was observed in HL-60 cells, whereas in HP100 cells, no DNA ladders were observed. In addition, the content of 8-oxodG of DBA in HL-60 cells treated with 20 μM catechol was significantly increased in comparison with non-treated cells. These results suggested that the catechol treatment generated H_2O_2 to induce 8-oxodG formation preceding apoptosis.

Different plant phenolics with catechol nuclei, such as caffeic acid, protocatechuic acid and chlorogenic acid stimulate the DNA degradation induced by Fenton reagents, e.g. Fe (III) and bleomycin [95-96]. Lévay et al. [97] have shown that the neurotransmitter dopamine which has a catechol nucleus and is found as an endogenous compound in plants and mammals caused DNA damage in two different ways: DNA adducts are formed and oxidative DNA damage upon incubation with cooper occurs [97].

It is well-known that catechol has strong promotion activity. Many investigations have indicated that catechol strongly enhances cancer development in rats and mice initiated with carcinogens, such as benzo[a]pyrene and N-methyl-N'-nitro-N-nitrosoguanidine [61-67]. Recent observations have suggested that some tumor promoters act to produce DNA damage mediated by reactive oxygen species [98-102]. Oikawa et al. [89] has demonstrated that catechol could induce metal-dependent H_2O_2 generation and subsequent damage to DNA fragments obtained from the human p53 and p16 tumor suppressor genes. Catechol also inhibits the rate limiting step of DNA synthesis in human T lymphoblasts, probably by inhibiting the ribonucleotide reductase. A covalent binding of catechol to the protein does not cause this inhibition or a protein cross-linking as the addition of $FeCl_3$ reverses the effect [103]. Compound with catechol moieties and a benzenic ring in their composition, such as catechol estrogens, L-DOPA, dopamine and α-methyl-DOPA can also cross-link proteins [104-107], for example, L-DOPA and dopamine cross-link neurofilaments. Thus, substances with the catecholic groups can cause DNA damage *in vitro* or *in vivo,* upon activation, for example, by heavy metals, or by cellular metabolism and conjugation reactions. DNA damage is either due to DNA-adduct formation by the catechols or their reaction intermediates or it is due to the formation of ROS causing an oxidation of DNA bases and/or DNA strand breaks.

Previous studies have been shown that the autoxidation products of apomorphine (APO) and other catechols (e.g. dopamine) might lead to deleterious effects on neuronal cells and neural function [108-109], and the cytotoxic effects of APO to cultured neurons have been shown to correlate to its autoxidation products. Picada et al. [110] showed a significant increase in DNA damage index and damage frequency in mice brain tissue 1 and 3 h after treatment with 8-OASQ, an autoxidation product of APO. Indeed, it has been demonstrated that 8-OASQ displays a higher frameshit mutagenic activity, which stimulates DNA strand breaks, when compared to APO [111]. At this same study, OASQ displays biological effects through the usual redox reactions of quinones and semiquinones generating H_2O_2 or O_2^- in addition to quinone and semiquinone radicals [112]. These reactions could promote an increased formation of $^{\bullet}OH$ radicals that are able to induce mostly single-strand breaks and various species of oxidized purines and pyrimidines [113-114].

The DNA damaged caused by catechol and hydroquinone containinng fraction of aqueous cigarette tar (ACT) has also been investigated for its DNA damaging activity in in vitro experiments. Hydrogen peroxide, superoxide and hydroxyl radicals are produced by this fraction and cause oxidative DNA damage [115]. This ACT also contains the tar radical, consisting of polymerized catechol, suggesting that catechol is involved in the production of the ROS. The tar radicals bind to DNA and DNA adducts are formed [116].

The DNA damaging activity of catechols estrogens (CE) has been intensively studied. During the redox cycling of CEs, in the presence of cooper (II), semiquinones and reactive oxygen species are formed and an increased rate of oxidative DNA damage can be measured [117-118]. Semiquinones and quinones formed from CEs can also damage DNA directly by covalent binding to the DNA [119-120].

Catechol toxicity is mainly connected with damage to protein and generation of hydrogen peroxide, which is capable to further failures. As a result of oxygen depletion, hydrogen peroxide is formed in the following reaction (oxygen consumption was stoichiometric with H_2O_2, production consistent with the reaction):

$QH_2 + O2 = H_2O_2 + Q$, where QH_2 is the catechol and Q is the *o*-quinone [121]. The above mechanism is confirmed also by Lee and Lin [122]. Catechol can produce H_2O_2, but cannot produce $O_2^{\cdot}$ and • OH [122]. The level of H_2O_2 generation of catechol plays an important role in the carcinogenic process induced by catechol and benzene [89]. Additionaly H_2O_2 is created during two-electron reduction of O_2 and also in the reaction of dismutation of O_2^{-} with the participation of SOD (superoxide dismutase). Catechol disturbs CAT and thus leads to promotion of higher amounts of H_2O_2. It seems that the observed decrease of the activity of superoxide dismutase in erythrocytes incubated with catechol is very important. Disturbing removal of superoxide radical (formed in high amounts during oxidation of HbO_2 to met-Hb) provoke further disturbance of catechol and the increase of the level of H_2O_2.

1.3- Reactive Oxygen Species and DNA Damage

Catechol binds covalently to cellular molecules, proteins and DNA in tissues. This binding is implicated in mechanisms of toxicity (inhibiting cell replication) and carcinogenicity (inititation of leukemia; 5). It is also supposed to act as a mutagen via an indirect mechanism, leading to oxidative DNA damage through the formation of hydroxyl radicals via hydrogen peroxide [123].

Briefly, reactive oxygen species are a family of small but highly reactive molecules, including singlet oxygen, superoxide anion, hydrogen peroxide, organic peroxide radicals and nitric oxide. ROS can be produced in vivo by many enzyme systems, including NADPH oxidase [1,2], NADH oxidase [124], xanthine oxidase [125], 5-lipoxygenase [126], and others. Under physiological conditions, the mitochondrial respiratory transport chain is the major site for ROS production in cells [127-128]. Based on studies in isolated mitochondria, about 1 to 2% of the oxygen consumed by the respiratory chain can be converted to ROS in the mitochondria [128].

Excessive production of ROS in the cell can be induced by a number of xenobiotics, transition metal ions, and ultraviolet and ionizing radiations. Indeed, under these conditions of increased ROS generation, or conditions of impaired antioxidant defense system, ROS may accumulate, exerting a potent damaging effect on the cell and the whole organism [129-134]. Mitochondria, being the main site of ROS generation in the cell, are also their primary target. This, in turn, results in damage to the mitochondrial respiratory chain and, as a consequence, a further increase in ROS production. A vicious cycle is thus formed [135] that may be a causative agent of number of age-associated dysfunctions of mitochondria and also one of the mechanisms inducing programmed cell death. Li et al. [136] have investigated the role of mitochondrial ROS in diphenyleneiodonium (DPI)-induced apoptosis, and showed that mitochondrial ROS could itself induce apoptosis via induction of mitochondrial membrane permeability, release of cytochrome c, and ativation of caspase 3 (factors that lead to apoptosis). In sum, the noxious action of ROS mainly consists of the peroxidation of lipids, in particular phospholipids of biological membranes, and oxidative damage to proteins and DNA [130, 133, 137].

It is now recognized that free radicals, including ROS, play a large role in many cellular processes, and the cell contains mechanisms to balance radical production and radical detoxification. Oxidative stress, however, can occur when this balance somehow becomes disturbed. Increased oxidative stress has been implicated in over 100 diseases, including ischemia/reperfusion injury, cancer, inflammation, degenerative diseases and aging [129]. One mechanism the cell uses to combat increased oxidative stress is the antioxidant enzyme, catalase, which is a heme protein found in the cytoplasm and peroxisomes. Catalase removes hydrogen peroxide from the cell by catalyzing its conversion to water. If not detoxified, hydrogen peroxide can interact with iron to form highly reactive radicals, which can initiate serious toxic reactions that can irreversible damage essential macromolecule targets [37].

Excessive generation of reactive oxygen species is presumed to be a significant factor in tissue injury observed in many disease states. There are numerous site of ROS production within cells including cytochrome P450 enzymes, xanthine oxidase, and the mitochondrial electron transport chain. It has been shown that mitochondria generate superoxide and hydrogen peroxide during state 4 respiration at levels which constitute about 2% of total mitochondrial oxygen uptake. Mitochondrial production of ROS is due to reduction of molecular oxygens that “leaks” from the unstable ubiquinone semiquinone that is formed during cycling redox of ubiquinone present in mitochondrial complex III [138]. Studies done by Barreto et al. [59] demonstrated that catechol inhibits liver mitochondrial state 2 $FADH_2$-linked respiration. These data suggest the inhibition of mitochondrial respiration by catechol as a potential mechanism of its cytotoxicity. Other resources have shown that endogenous catechols and exogenous molecules bearing a catechol moiety are also inhibitors of mitochondrial respiration. Endogenous cysteinylcatechols are potent inhibitors of mitochondrial complex I activity in vitro. Dopamine and its metabolite 3,4-

dihydroxyphenylacetic acid can inhibit brain mitochondrial state 3 NADH-linked respiration [139]. Flavonoids with a catechol on their rings are inhibitors of state 2 $FADH_2$ – and NADH-linked respiration [140] A previous study about catechol-*o*-methyltransferase inhibitors demonstrated the effects of 3,4-dihydroxy-4´-methyl-5-nitrobenzophenone (tolcapone) in decreasing respiratory control ratio in mitochondrial preparations at low micromolar concentrations [141]. Tolcapone has been associated with hepatotoxicity. Furthermore, tolcapone reduced ATP synthesis in human neuroblastoma SH-SY5Y cells [142]. From this data, it is presumibly that catechol inhibits mithocondrial respiration, and so leads to the formation of ROS and reactive quinones during its autoxidation. Thus, quinones may contribute to increase H_2O_2 production in mitochondria, as reported in subcellular fractions treated with estrone 3,4-quinone and NADPH [143-145].

In order to assembly and investigate the level of DNA damage, the comet assay has been widely used to quantify DNA lesions in mammalian cells. Even so, it can detect DNA strand breaks, alkali-labile sites and incomplete excision repair events in individual cells [46].

1.4- Evaluating the DNA Damage: The "Comet Assay"

The alkaline single-cell gel electrophoresis (SCG) assay, a new procedure for evaluating DNA lesions (single strand breaks and alkali-labile sites, DNA cross-linking and incomplete excision repair), involves application of an electrical current to cells, which results in the transport of DNA fragments out of the nucleus. The image of DNA migration obtained resembles a comet with a head and a tail, hence the term comet [146-147]. Since the DNA damage induced by toxic agents is often tissue-and-cell specific, SCG is very useful because it can detect DNA lesions in individual cells obtained under a variety conditions; the technique

has been shown to be a very sensitive method and a useful tool to detect genetic damage and DNA repair [148] at the individual cell level and in human biomonitoring [46, 149]. A significant advantage of the SCG assay is its applicability to any eucaryotic organism and cell type. Since the assay is also inexpensive and gives results within a few hours, it is appropriate for environmental monitoring. In addition to human peripheral blood lymphocytes exposed to different agents, both in vitro and in vivo [150], other cell types and organisms have also been tested with this assay [148, 151-154].

Many studies have reported the use of comet assay to evaluate DNA damage [155-159] and the main focus is based on long-term exposure to volatile compounds such as benzene and catechol as it main metabolite. Thus, Moretti et al. [158] investigated the effects of physical agents and benzene through the comet assay, concluding that this relation might interfere with the genotoxic activity of xenobiotics of exposed population.

In order to evaluate DNA damage, single cell gel electrophoresis, known as the 'comet assay', has assembly to be a potential way to quantitate the DNA damage in workers exposed to low levels of genotoxic agents since it is more sensitive than other methods [46, 123]. Andreoli et al. [123] showed that there was significantly higher DNA damage in the lymphocytes of subjects occupationally exposed to low levels of benzene compared with matched, unexposed controls. Sul et al. [35] showed that the comet assay provided some evidence that B-lymphocytes are more sensitive to low levels of benzene than are T-lymphocytes or granulocytes. This result could be ascribed to the greater genomic damage of the primary lymphopoietic organ, the bone marrow [1]. Thus, B-lymphocytes may be a more useful target than other blood cells for the biomonitoring of human exposure to low levels of benzene and possibly other genotoxic agents.

In the comet assay, cells embedded in the agarose on a microscope slide are lysed, leaving DNA embedded in the agarose. During electrophoresis, DNA migrates towards the anode. Cells with DNA damage (i.e. DNA with strand breaks) exhibit more migration and appear comet shaped with a circular head and an elongated tail; those without damage have no tail. The greater the damage, in the form of breaks, the longer and more intense the comet tail. Quantification of single-strand breaks, double-strand breaks and alkali-labile sites, respectively, are possible after lysis and electrophoresis under alkaline condition [160-161].

There are a number of ways in which the comet assay can be modified to provide insights on the specific types of damage to DNA. Oxidative damage can be detected using formamidopyrimidine DNA glycoslyase (Fpg) [150, 160, 162-163]. Oxidative DNA damage can be particularly harmful to cells. Normal or background levels of oxidative DNA damage are usually efficiently repaired. Exposure to certain contaminants can cause increased levels of oxidative DNA damage. When DNA is treated with Fpg during the comet assay, additional strand breaks can then be detected as an increase in DNA migration [164-165]

Phenolic molecules are widely present in the environment and some of them are well known carcinogens. Some phenolic molecules are also genotoxic but the mechanisms involved in this process are not fully understood. Catechol is implicated in reactive oxygen species production via generation of sequinones and reactive quinones. Thus, those molecules are more toxic than catechols. Benzene and its main metabolite, catechol, are supposed to play an important role in cytotoxicity and consequently leading to DNA damage. Although catechols has been extensively studied in many organic systems, it is necessary further investigations to clear up the mechanisms and pathways involved in toxicity and DNA damage as well.

CONCLUSIONS

We have reviewed the recent advanced studies regarding the benzene, and its main metebolite, catechol, in many biological systems. The key role and metabolization of catechol involved in the semiquinones and reactive quinones production, leading to reactive oxygen species generation is quite known. Some mechanisms involving ROS and oxidative DNA damage still remain unknown in certain aspects and are being a target of inumerous studies. Although there are few studies that indicate the harzadous effects of catechols, some others are necessary to identify further consequences in long term exposure to this molecule. Such information can be quite important for further pharmaceutical design of new drug carries for gene therapy.

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List of Abbreviations

ROS – Reactive Oxygen Species

DNA – Deoxyribonucleic Acid

CYP – Cytochrome P450

CYP2E1 – Cytochrome System

1,2-BD, catechol – 1,2-benzenediol

1,4-BD, hydroquinone – 1,4-benzenediol

1,2,4-BT – 1,2,4-benzenetriol

t,t-MA – Trans, trans-muconic acid

H_2O_2 – Hydrogen Peroxide

$^{\bullet}OH$ – Hydroxyl Radicals

8-oxodG – 8-hydroxy-2'-deoxyguanisine

APO – Apomorphine

8-OASQ – autoxidation product of APO

CE – Catechol Estrogens

SOD – Superoxide Dismutase

DPI – Diphenyleneiodonium

Tolcapone – 3,4-hydroxy-4'-methyl-5-nitrobenzophenone

SCG – Alkaline Single-cell Gel Electrophoresis (Comet Assay)

FPG – Formamidopyrimidine DNA Glycoslyase

List of Legends

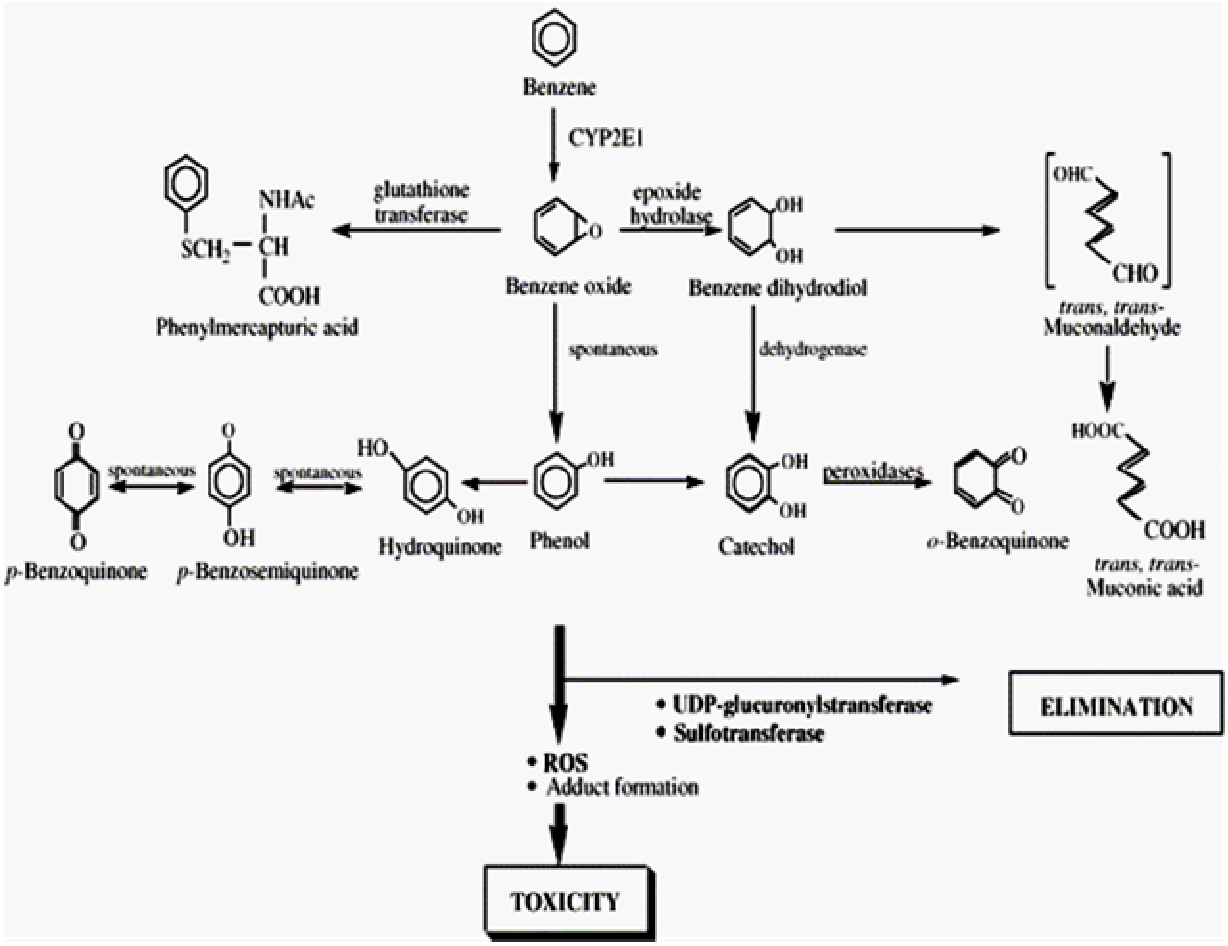


Fig. (1). Benzene Bioactivation pathway

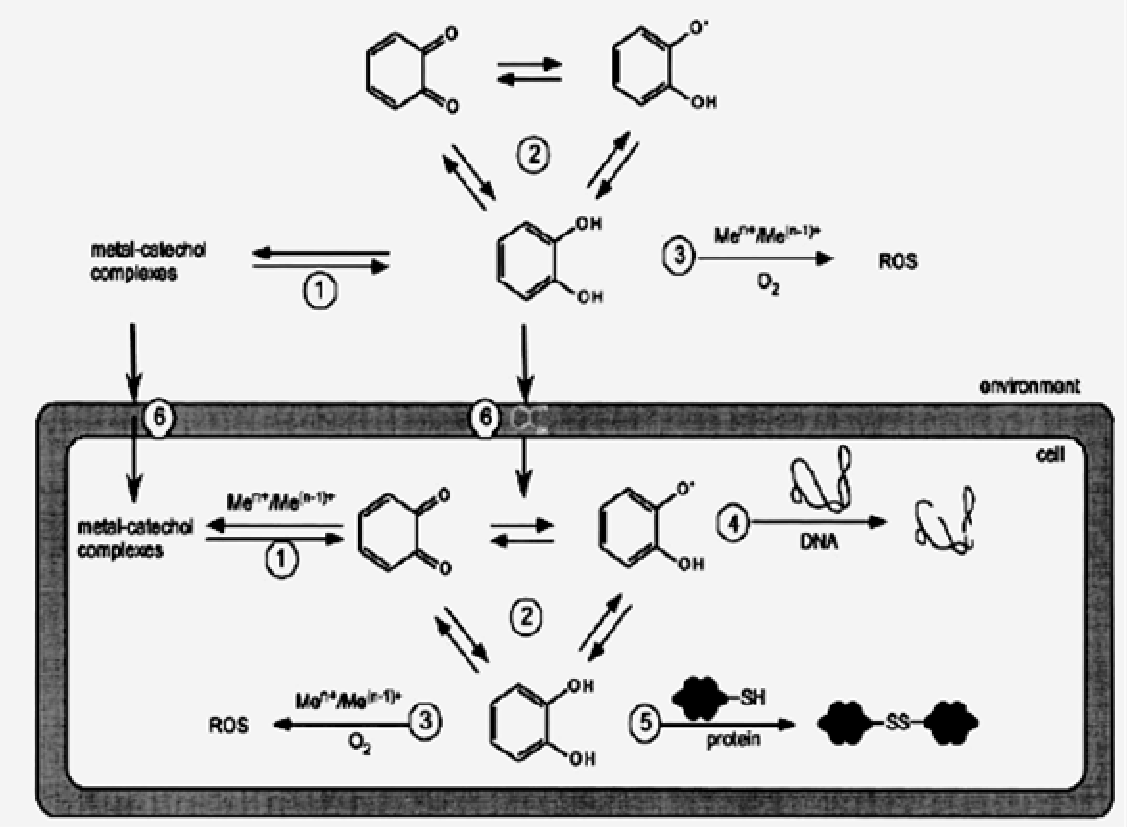


Fig. (2). Chemical reactions of catechols in the environment and in cells and molecular modes of action in cells. Chemical reactions: 1, Complex formation with heavy metals; 2, Redox cycling; 3, Production of reactive oxygen species (ROS) in the reaction with heavy metals and oxygen. Molecular modes of actions in cells: 4, DNA damage (for example, strand breaks and DNA adduct formation); 5, protein damage (for example, protein cross-linking via disulphide groups); 6, absorption in membranes and possible interactions [56].

3.2 Artigo II – Catechol inhibit $FADH_2$-linked respiration in rat liver mitochondrial fraction.

O benzeno é um composto volátil largamente estudado na atualidade. Apesar de diversos trabalhos enfatizarem os efeitos tóxicos desse hidrocarboneto, alguns mecanismos relacionados com a geração final de espécies reativas de oxigênio, precisam ser melhor elucidados. Sabe-se, portanto, que a formação de quinonas e semiquinonas reativas, culminando com a produção de EROs tem um papel importante na toxicidade do catecol.

Mediante essas insertivas, o objetivo deste trabalho foi testar a hipótese do catecol (pirocatecol) inibir a respiração basal associada ao $FADH_2$ em frações mitocondriais hepáticas de rato. Além disso, estudou-se também a capacidade do catecol de induzir peroxidação de biomoléculas nas frações nucleares.

Com esse estudo, evidenciou-se que o catecol induziu uma inibição parcial da respiração basal mitocondrial associada ao $FADH_2$ de forma dependente do tempo, contudo essa substância não induziu peroxidação direta das biomoléculas presentes nas frações nucleares hepáticas.

Este artigo foi publicado no Suplemento Especial do periódico **Acta Cirúrgica Brasileira** (Acta Cir Bras 2005; 20:72-77) que foi editado para ser distribuído durante o IX Congresso Nacional de Cirurgia Experimental – SOBRADPEC e I Simpósio Nacional de Pós-Graduação em Ciências da Saúde – UFRN.

A cópia definitiva deste artigo publicada no Sistema Scielo (**Acta Cir Bras** 2005; 20:40-45) encontra-se nas próximas páginas (53 a 65).

Catechol inhibits $FADH_2$-linked respiration in rat liver mitochondrial fraction[1]

Catecol inibe a respiração ligada ao $FADH_2$ na fração mitochondrial do fígado do rato

George Emílio Sampaio Barreto[I]; Gleide Souza dos Santos[II]; Eryvaldo Sócrates Tabosa Egito[III]; Ramon dos Santos El-Bachá[IV]

[I]Physiotherapist, specialist in Respiratory Care, M. Sc. student of the Graduation Program in Health Sciences, Laboratory of Dispersed Systems (LASID), Department of Pharmacy, Federal University of Rio Grande do Norte (UFRN), Natal – RN - Brazil
[II]Undergraduate student of Nursing, UFBA, Fellowship of FAPESB, LabNq, ICS,UFBA, Salvador - BA - Brazil
[III]Ph. D., Head of Pharmacy Course, Head of LASID, Department of Pharmacy-UFRN, Professor of Postgraduate Program of Health Sciences-UFRN, Natal - RN - Brazil
[IV]Ph. D., Head of LabNq, Department of Biochemistry and Biophysics, ICS, UFBA, Salvador – BA - Brazil

ABSTRACT

PURPOSE: The aim of this work was to investigate the hypothesis that catechol inhibits $FADH_2$-linked basal respiration in mitochondria isolated from rat liver homogenates. Moreover, catechol ability to induce peroxidation of biomolecules in liver nuclear fractions was also studied.
METHODS: Rat liver homogenates were incubated with 1mM 1,2-dihydroxybenzene (catechol) at pH 7.4 for up to 30 minutes. After that, mitochondrial fractions were isolated by differential centrifugation. Basal oxygen uptake was measured using a Clark-type electrode after the addition of 10 mM sodium succinate. Nuclear fractions were incubated in the presence of 1 mM catechol for 17 hours at room temperature and the peroxidation of biomolecules was investigated by the reaction with thiobarbituric acid, which was determined spectrophotometrically at 535 nm.
RESULTS: Catechol induced a time-dependent partial inhibition of $FADH_2$-linked basal mitochondrial respiration, however this substance was unable to induce a direct peroxidation of biomolecules in hepatic nuclear fractions.
CONCLUSION: Catechol produced an inhibition of basal respiration associated to $FADH_2$ in isolated liver mitochondria that could lead to cytotoxicity, ROS generation and cell death.

Keywords: Catechol. Mitochondrial respiration. Peroxidation.

RESUMO

OBJETIVO: Testar a hipótese do catecol inibir a respiração basal associada ao $FADH_2$ em frações mitocondriais hepáticas de rato. Além disso, estudou-se também a capacidade do catecol de induzir peroxidação de biomoléculas nas frações nucleares.
MÉTODOS: Os homogeneizados de fígado de ratos foram incubados com catecol a 1 mM em pH fisiológico. Depois disso, as frações mitocondriais foram isoladas por centrifugação diferencial. O consumo basal de oxigênio foi medido com um eletrodo do tipo Clark após injeção de succinato a 10 mM. Frações nucleares foram incubadas com catecol por 17 horas à temperatura ambiente e a peroxidação de biomoléculas foi investigada pela reação com o ácido tiobarbitúrico e mensurada espectrofotometricamente.
RESULTADOS: O catecol induziu uma inibição parcial da respiração basal mitocondrial associada ao $FADH_2$ de forma dependente do tempo, contudo essa substância não induziu peroxidação direta das biomoléculas presentes nas frações nucleares hepáticas.
CONCLUSÃO: O catecol produz inibição da respiração basal associada ao $FADH_2$ em mitocôndrias isoladas de fígado, o que pode levar à toxicidade, produção de espécies reativas e morte celular.

Descritores: Catecol. Respiração mitocondrial. Peroxidação.

Introduction

Benzene is an ubiquitous environmental chemical that is used as a precursor in the synthesis of numerous products including drugs, dyes, insecticides, and plastics. This compound is also found in unleaded gasoline, cigarette smoke and industrial emissions [1]. Based on epidemiological studies, human exposure to benzene causes bone marrow depression, acute myelogenous leukemia, acute lymphocytic leukemia, myelotoxicity, non-Hodgkin´s lymphoma, lung cancer and nasopharyngeal cancer [1,2]. The liver is the primary site of benzene metabolism and the major metabolic enzyme system is cytochrome P_{450} (primarily CYP2E1). This involves the formation of a series of reactive metabolites like phenol, hydroquinone and 1,2-dihydroxybenzene (catechol) [2].

Catechols are constituted by a large group of compounds from natural or synthetic origin, all them containing the common 1,2-dihydroxybenzene ring. They are used in a variety of applications, such as a reagent for photography, dyes, rubber and plastic production, and in the pharmaceutical industry [3]. Catechols are intermediary products

from the degradation of aromatic compounds. In humans and other mammals, catechols can occur as metabolites in the degradation of benzene and estrogens[4].

Catechols readily undergo oxidation to form semiquinone radicals and quinones, which are more reactive than catechols. The mechanisms most frequently cited to explain the toxicity of catechols are: (i) the generation of reactive oxygen species (ROS) by redox reactions; (ii) DNA damage in the form of oxidative damage or DNA arylation; (iii) protein damage by sulfhydryl arylation or oxidation; and (iv) interference with electron transport in energy transducing membranes [5]. Moreover, catechol releases iron from ferritin inducing lipid peroxidation in brain homogenates [6]. When catechol is oxidized enzimatically or in the presence of oxygen and heavy metals, one electron is transferred to the molecular oxygen, and consequently superoxide (O_2^-) is formed. In the presence of heavy metals (e.g. copper, iron), superoxide is further reduced to hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH). These ROS can be harmful to cells and organisms if they are not eliminated [7]. Catechol can act as pro-oxidant damaging macromolecules such as DNA and proteins, and destroying membrane functioning due to their redox cycling activity. Our laboratory recently reported that catechol-induced cytotoxicity towards glioblastoma cells in vitro is due to the production of superoxide and reactive quinones [8].

Although the exact mechanism for benzene-induced toxicity is unknown, it seems that catechol plays an important role regarding the effects of the exposure to this molecule. The lethal human dose of catechol is 454 - 4540 mmol/kg or one teaspoon given once for a 70 kg person. Therefore, in the present study we investigate the toxic effect of 1 mM catechol towards liver subcellular fractions since this organ is responsible for xenobiotic metabolism. This concentration is in the range of the catechol level found accumulated in people exposed to action of this compound (90 - 9000 μM) [9]. The hypothesis that catechol inhibits mitochondrial state 2 $FADH_2$-linked respiration that could be a mechanism of toxicity was tested. Furthermore, the ability of catechol to

induce an oxidative stress and a direct peroxidation of biomolecules due to its autoxidation was assessed.

Methods

Animals

Adult Wistar rats weighing 250-350 g were obtained from the Department of Physiology of the Health Sciences Institute of the Federal University of Bahia (Salvador, BA, Brazil). All experimental protocols were conducted according to regulations suggested by the Federal University of Bahia Ethical Committee.

Mitochondrial isolation

Mitochondria were isolated from liver of adult rats by differential centrifugation. Liver was homogenized in 200 mM mannitol, 75 mM sucrose, 1 mM ethylene glycol-bis[b-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA), 0.05% (w/v) bovine serum albumin (BSA) and 1 mM Tris buffer (pH 7.4) using a teflon pestle tissue homogenizer. Homogenates were incubated in the absence (controls) or in the presence of 1 mM catechol for 0, 15 or 30 minutes at 37 °C before centrifugation. Two independent experiments were done for each incubation. Whole cells, nuclei, cytoskeletons and plasma membranes were removed by centrifugation at 550 ***g*** for 10 minutes at 4 °C, followed by centrifugation of the supernatant at 7,100 ***g*** for 10 minutes at the same temperature. The mitochondrial pellet was resuspended in the same buffer and washed twice at 6,400 **g** for 10 minutes at 4 °C. Finally, mitochondria were resuspended in 1 ml of the same isolation buffer. Protein determinations were performed according to Lowry [10].

Oxygen electrode measurements

Oxygen consumption was carried out at 37 °C in a closed chamber containing a Clark type oxygen electrode connected to a YSI model 53 monitor (Yellow Springs Instrument Co. Inc., OH, USA). Isolated mitochondria were suspended in 3 ml of 10 mM KCl, 0.2 mM ethylenediamine tetraacetic acid (EDTA), 0.25 M mannitol, 0.025% (w/v) BSA and 10 mM Tris, 5 mM phosphate buffer (pH 7.4) at a final concentration of 0.4 mg protein/ml. Sodium succinate was added to a final concentration of 10 mM in order to induce mitochondrial basal respiration (state 2). Oxygen uptake in resting conditions was monitored for 12 minutes. Four assays were analyzed for each mitochondrial fraction.

Isolation of nuclear fraction

Nuclear fraction was isolated from liver of adult rats. To isolate nuclei, liver was weighed and suspended (0.3g/ml) in 0.25 M sucrose, 10 mM EDTA and 50 mM phosphate buffer (pH 7.4). The tissue was homogenized on ice using a teflon pestle tissue homogenizer. Cells were lysed with 8 up-and-down pestle strokes. Tissue homogenate was centrifuged at 400*g* for 10 minutes at 4 °C and the supernatant was stored at 4 °C. The pellet was resuspended in the same buffer and centrifuged again in the same conditions. Supernatants were combined and centrifuged at 1,500*g* for 10 minutes at 4 °C. Nuclei were resuspended in 1 mM EDTA, 20% glycerol and 100 mM phosphate buffer (pH 7.4). Protein concentrations were determined as described for mitochondrial isolation.

Peroxidation assay

Nuclear fractions (1 mg/ml) were incubated with 1 mM catechol in 1 mM HCl and 50 mM phosphate buffer (pH 7.4) at room temperature for 17 hours. Negative controls were incubated in the absence of catechol and positive controls were incubated in the

presence of 5 mM $FeSO_4$ and 500 mM ascorbate. Peroxidation was assessed by measuring the formation of thiobarbituric acid-reactive substances (TBARS) as described previously [11]. A great variety of oxidized substances form pink TBA complexes, such as malonaldehyde, oxidized sugars and amino acids [12], and all these compounds can be found in nuclear fractions submitted to an oxidative stress. After addition of 2 ml of 0.67% (w/v) TBA, 15% (w/v) trichloroacetic acid in 0.25 M HCl, samples were boiled in capped tubes for 10 minutes and cooled on fresh water. The reaction mixture was centrifuged at 1,500 ***g*** for 10 minutes. The optical density was measured against a blank without TBA in a Micronal B 382 spectrophotometer at 535 nm .

Statistics

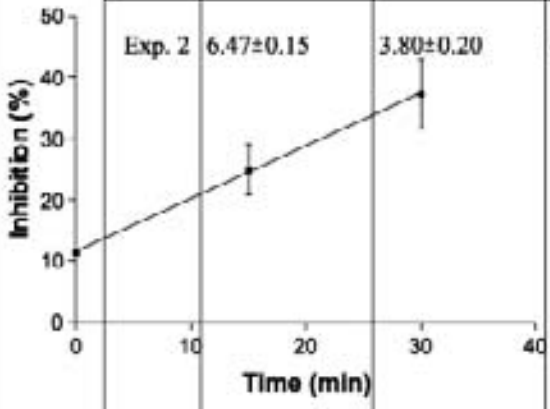
Samples and controls were compared by the Student's *t* test. Significant differences were considered for $P < 0.05$. Pearson's test was used to correlate catechol-induced inhibition of mitochondrial respiration with time.

Results

Catechol inhibited mitochondrial state 2 $FADH_2$-linked respiration when liver homogenates were incubated in the presence of this compound (Table 1). The inhibition (11.5%) was evident even when mitochondrial fractions were isolated just after the addition of 1 mM catechol (Time 0). The inhibition increased when homogenates were incubated with this molecule for 15 minutes (25 %) or 30 minutes (37.3%) before mitochondrial isolation. Moreover, the inhibition linearly correlates with time (Pearson's correlation, 0.96458; $P < 0.002$; Figure 1).

Table 1. Inhibition of liver mitochondrial state 2 FADH -linked respiration by 1 mM catechol. Liver homogenates were incubated in the absence (controls) or in the presence of 1 mM catechol for times ranging between 0 - 30 minutes prior to mitochondrial isolation. Two independent experiments were carried out for each incubation. Four assays of oxygen uptake were analyzed in each experiment. Data are expressed as mean ± SD.

TIME (min)		Catechol 0 mM	Catechol 1mM	% Inhibition	P
Time 0	Exp. 1	8.48±0.45	7.49±0.34	11.7	< 0.02
	Exp. 2	8.01±0.44	7.11±0.5	11.2	< 0.05
Time 15	Exp. 1	2.50±0.09	1.95±0.15	22	< 0.01
	Exp. 2	4.55±0.24	3.28±0.3	27.9	< 0.001
Time 30	Exp. 1	4.9±0.16	3.27±0.24	33.3	< 0.0001
	Exp. 2	6.47±0.15	3.80±0.20	41.3	< 0.0001



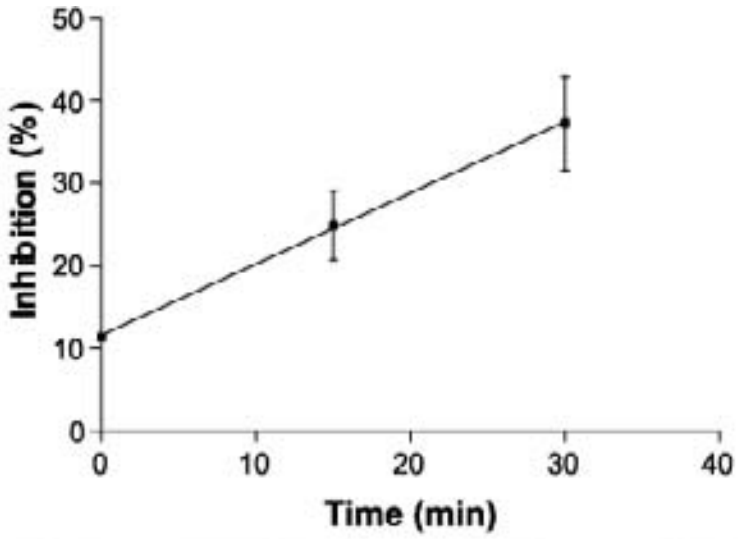


FIGURE 1 – Correlation between the inhibition of liver mitochondrial state 2 FADH -linked respiration and the time of incubation of liver homogenates in the presence of 1 mM catechol prior to mitochondrial isolation (Pearson's correlation, 0.96458; $P < 0.002$)

Table 1. Inhibition of liver mitochondrial state 2 $FADH_2$-linked respiration by 1 mM catechol. Liver homogenates were incubated in the absence (controls) or in the presence of 1 mM catechol for times ranging between 0 - 30 minutes prior to mitochondrial isolation. Two independent experiments were carried out for each incubation. Four assays of oxygen uptake were analyzed in each experiment. Data are expressed as mean ± SD.

In order to investigate if catechol could induce a direct peroxidation of biomolecules, nuclear fractions were incubated with this substance at 1 mM for 17 hours at room temperature and TBARS were measured after this. Catechol did not induce peroxidation of biomolecules present in nuclear fractions (Figure 2). On the other hand, a marked peroxidation was observed in samples incubated in the presence of ascorbate and $FeSO_4$ (positive controls).

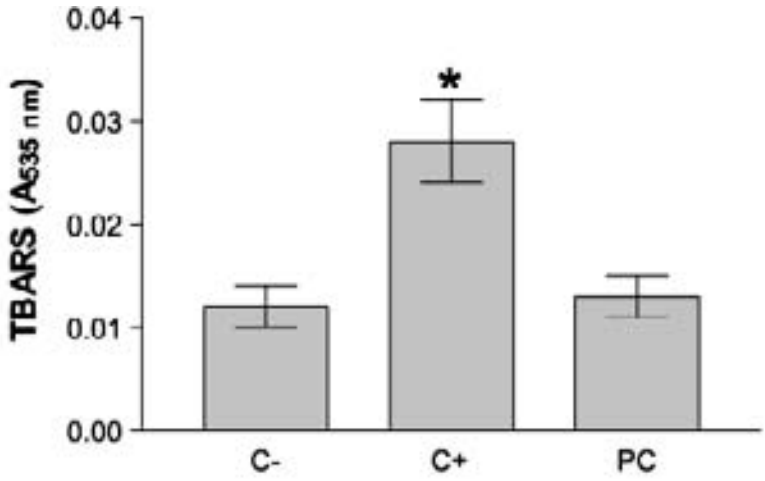


FIGURE 2 – Peroxidation of biomolecules that are present in liver nuclear fractions. Nuclear fractions were incubated in the absence (C-) or in the presence of 1 mM catechol (PC) for 17 hours at room temperature. Positive controls (C+) were incubated in the presence of 5 mM $FeSO_4$ and 500 mM ascorbate at the same conditions. Afterward, peroxidation was assessed by measuring the formation of TBARS which absorbs light at 535 nm. Data are expressed as mean ± SD. *, $P < 0.05$ compared to C-.

Discussion

Data from the present study demonstrate that catechol inhibits liver mitochondrial state 2 $FADH_2$-linked respiration. These data suggest the inhibition of mitochondrial respiration by catechol as a potential mechanism of its cytotoxicity. Other laboratories have shown that endogenous catechols and exogenous molecules bearing a catechol moiety are also inhibitors of mitochondrial respiration. Endogenous cysteinylcatechols are potent inhibitors of mitochondrial complex I activity in vitro [13]. Dopamine and its metabolite 3,4-dihydroxyphenylacetic acid can inhibit brain mitochondrial state 3 NADH-linked respiration [14]. Flavonoids with a catechol on their rings were inhibitors of state 2 $FADH_2$- and NADH-linked respiration [15]. A previous study about catechol-O-methyltransferase inhibitors demonstrated the effects of 3,4-dihydroxy-4'-methyl-5-nitrobenzophenone (tolcapone) in decreasing respiratory control ratio in mitochondrial preparations at low micromolar concentrations [16]. Tolcapone has been associated with hepatotoxicity. Furthermore, tolcapone reduced ATP synthesis in human neuroblastoma SH-SY5Y cells [17].

Excessive generation of reactive oxygen species (ROS) is presumed to be a significant factor in tissue injury observed in many disease states. There are numerous sites of ROS production within cells including cytochrome P450 enzymes, xanthine oxidase, and the mitochondrial electron transport chain. It has been shown that mitochondria generate superoxide and hydrogen peroxide during state 4 respiration at levels which constitute about 2% of total mitochondrial oxygen uptake. Mitochondrial production of ROS is due to reduction of molecular oxygen by an electron that "leaks" from the unstable ubiquinone semiquinone anion that is formed during redox cycling of ubiquinone present in mitochondrial complex III [18]. Furthermore, mitochondrial ROS may inhibit one or more of the components of the respiratory chain, further accelerating the rate of superoxide formation. The molecular targets of mitochondrial-derived ROS have not been clearly established, although lipid peroxidation, ion channel modification and DNA damage have all been demonstrated in models where the effects of exogenous oxidizing agents have been studied [19].

Catechol leads to the formation of ROS and reactive quinones during its autoxidation. Quinones may contribute to increase H_2O_2 production in mitochondria, as reported in subcellular fractions treated with estrone 3,4-quinone and NADPH [20]. Oxidative stress increases the opening of mitochondrial permeability transition pore (mPTP) and depolarisation [21], suggesting that this mechanism may be important in cell death induced by catechol.

In our experiments catechol did not induce a direct peroxidation of biomolecules in liver nuclear fractions. Indeed, other authors showed that catechols such as catechol estrogens, catecholamines, catechins and caffeic acid strongly inhibit lipid peroxidation [22]. However, catechol releases iron from ferritin inducing lipid peroxidation in brain homogenates [6]. Although the pro-oxidant or antioxidant properties of catechols rests a controversial subject, our data show that the inhibition of mitochondrial respiration induced by catechol is more important than the peroxidation of biomolecules in liver subcellular fractions.

Benzene is metabolized in the liver to catechol by hepatic cytochrome P450 enzymes, particularly CYP2E1 [23-24]. Exposure to benzene has been associated with perisinusoidal fibrosis, histological evidence of cholestasis, and non-alcoholic steatohepatitis [25]. Overall, the results from this study permit us to propose that benzene toxicity is related to formation of catechol which kills hepatocytes by inhibiting $FADH_2$-linked mitochondrial respiration.

Conclusion

The catechol level found accumulated in people exposed to action of this compound is in the range of 90 - 9000 μM. This study showed that catechol at 1 mM inhibits state 2 $FADH_2$-linked mitochondrial respiration. However, this molecule did not induce peroxidation in nuclear fractions. These data suggest that catechols may exert hepatotoxicity by inhibiting mitochondrial respiration. This mechanism may be related to benzene-induced non-alcoholic steatohepatitis, since catechol is one of its main metabolites.

Acknowledgments

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Correspondence to
Ramon dos santos El-Bachá
Mailing address: ramon@ufba.br.

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1 Laboratory of Neurochemistry and Cell Biology (LabNq), Health Sciences Institute (ICS), Federal University of Bahia (UFBA), Salvador - BA - Brazil.

3.3 Artigo III – Investigation of toxic factors affecting cells of rat brain exposed to 3-methylcatechol.

O 3-metilcatecol (3MC) é um derivado do tolueno. Estudos demonstram sua toxicidade em alguns sistema orgânicos, principalmente em estudos *in vitro* e *in vivo*. Para elucidar os potenciais efeitos tóxicos desse composto no SNC, procuramos analisar os efeitos do 3MC na respiração mitocondrial a partir de mitocôndrias cerebrais de ratos.

Preliminarmente, o 3MC inibiu o Complexo II da respiração mitocondrial ligada ao $FADH_2$. Este resultado entra em concordância com os já encontrados em frações hepáticas, inclusive utilizando-se o pirocatecol como substância-teste.

Este artigo foi submetido ao periódico **Brazilian Archives of Biology and Technology**, e a cópia encontra-se nas páginas 67 a 79.

Investigation of toxic factors affecting cells of rat brains exposed to 3-methylcatechol

George Emílio Sampaio Barreto[1,3], Elineusa Silva de Oliveira[1], Flávio Augusto Guerreiro Aragão de Villar[1], Gleide Souza dos Santos[1], Rute Maria Ferreira Lima[1], Mírian Rocha Vázquez[2], Silvia Lima Costa[1], Eryvaldo Sócrates Tabosa Egito[4] and Ramon dos Santos El-Bachá[1*]

[1] Laboratório de Neuroquímica e Biologia Celular; Departamento de Biofunção; Instituto de Ciências da Saúde; Universidade Federal da Bahia; Campus do Canela; 40110-100 Salvador – Bahia – Brasil, [2] Departamento de Ciências da Vida; Centro de Ciências da Saúde e dos Alimentos, Universidade do Estado da Bahia; Salvador – Bahia – Brasil, [3] Programa de Pós-Graduação em Ciências da Saúde; Universidade Federal do Rio Grande do Norte (UFRN), Natal –Rio Grande do Norte – Brasil, [4] Laboratório de Sistemas Dispersos; Departamento de Farmácia, UFRN, Natal – Rio Grande do Norte - Brasil

ABSTRACT

3-Methylcatechol (3MC) is a metabolite of toluene. To clarify whether 3MC could be toxic towards the central nervous system, we examined the effects of 3MC on the peroxidation of biomolecules in nuclear fractions and mitochondrial respiration in organelles obtained from rat brains. We also tested the cytotoxicity towards rat primary astrocytes in vitro. 3MC at 1mM oxidizes consuming oxygen with a rate of 1.98 ± 0.19 $\mu M.min^{-1}$ and forming reactive quinones. At this same concentration 3MC induced peroxidation of biomolecules in nuclear fractions obtained from rat brain homogenates. This compound also inhibited state 2 $FADH_2$-linked mitochondrial respiration. Furthermore, 3MC also oxidizes in the culture medium leading to the formation of quinones. This toluene metabolite was cytotoxic towards rat primary astrocytes. The concentration that killed 50% of cells after 72 hours was 107 μM. The results of the study indicate a direct relationship between the cytotoxicity and the oxidation of 3MC.

Key words: Astrocytes, brain, cytotoxicity, 3-methylcatechol, peroxidation, respiration

[*] Correspondence author. Tel.: 55 71 32 45 86 02; Fax: 55 71 32 45 89 17.

E-mail address: ramon@ufba.br

INTRODUCTION

Inert organic solvents such as benzene, toluene, ethylbenzene and xylene (BTEX) have been used by the chemical industry to prepare solid catalysts (Tashino et al., 2005), for polymerization reactions (Shikuma et al., 2005), to dissolve photosensitive compositions (Ishizuka and Kojima, 2005), to synthesize pesticidal compositions (Mori, 2005), pharmaceutical compositions (Watanabe et al., 2005), and others. BTEX represent an estimated 35% of fuel oils. Moreover, benzene and toluene are also gas phase tobacco smoke constituents (Xue et al., 2005). Since these compounds are recognized hazardous fuels and solvents, methods for determination of aromatic hydrocarbons have been developed for toxicity assessment and confirmation of environmental contamination (Dijke, 2005). Inhalation of BTEX poses a health concern due to high volumes of production and release into the atmosphere.

Biological transformation and volatilization are the major removal mechanisms for toluene contamination of soils and groundwater. Toluene is a substrate for wild or modified toluene dioxygenases expressed in prokaryotic cells leading to the formation of 3- and 4-methylcatechols (Arnold et al., 2005). In rats and humans, the P450 2E1 content is an important parameter in the hepatic clearance of toluene (Béliveau et al., 2005). Several lines of evidence suggest that the acute neurotoxicity of toluene results from specific receptor-mediated interactions between this solvent and ion channel proteins (Bushnell et al., 2005). Although the toxicity of toluene is well recognized, the cytotoxicity of its metabolites has not been studied extensively. 3-Methylcatechol (3MC) is a minor metabolite of toluene in human, but studies have reported that 3MC is cytotoxic. Hela cells exposed to 3MC showed an inhibition of cell growth and a dose-related decrease in cell viability and cell protein content (Shen, 1998). Furthermore, 3MC induced oxidative DNA damage in rat testis (Nakai et al., 2003).

To clarify whether 3MC could be toxic towards the central nervous system, we examined the effects of this compound on the peroxidation of biomolecules in nuclear fractions obtained from rat brains. To determine if 3MC could interfere on brain mitochondrial function, we examined the effects of this molecule on $FADH_2$-linked basal mitochondrial respiration. Furthermore, we investigated 3MC-induced cytotoxicity towards rat primary astrocytes in vitro.

All reagents used in this work were analytical grade.

Measurement of 3-methylcatechol autoxidation in a cell-free system

Neutral aqueous solutions of 3MC undergo spontaneous oxidative decomposition. Autoxidation is a multi-step reaction resulting in reactive oxygen species (ROS) and quinone derivatives formation. In the present study, the autoxidation rates of 1 mM 3-methylcatechol were measured at 320 nm in 1 mM HCl, 50 mM phosphate buffer (pH 7.4) in the absence or in the presence of 17 U superoxide dismutase (EC

1.15.1.1). 3-Methylcatechol oxidation was also measured by means of a Clark-type oxygen electrode fitted to an oxygen monitoring system.

Animals

One-day-old and adult Wistar rats weighing 250-350 g were obtained from the Department of Physiology of the Health Sciences Institute of the Federal University of Bahia (Salvador, BA, Brazil). All experimental protocols were conducted according to regulations suggested by the Federal University of Bahia Ethical Committee.

Isolation of nuclear fraction

Nuclear fraction was isolated from brains of adult rats. To isolate nuclei, three brains were homogenized using a ground-glass pestle in 25 ml of 0.32 M sucrose, 1 mM ethylenediamine tetraacetic acid (EDTA), 50 mM phosphate buffer (pH 7.4), on ice. Cells were lysed with 8 up-and-down pestle strokes. Tissue homogenate was centrifuged at 400 ***g*** for 10 minutes at 4 °C and the supernatant was stocked at 4 °C. The pellet was resuspended in the same buffer and centrifuged again in same conditions. Supernatants were combined and centrifuged at 1,500 ***g*** for 10 minutes at 4 °C. The nuclei were resuspended in 0.2 M EDTA, 20% glycerol, 0.1 M phosphate buffer (pH 7.4). Protein concentrations were determined according to Lowry et al. (Lowry et al., 1951).

Peroxidation assay

Nuclear fractions (1 mg protein/ml) were incubated with 1 mM 3-methylcatechol in 1 mM HCl, 50 mM phosphate buffer (pH 7.4) at room temperature for 17 hours. Negative controls were incubated in the absence of 3MC and positive controls were incubated in the presence of 5 µM Fe_2SO_4 and 0.5 mM ascorbate. The formation of thiobarbituric acid (TBA)-reactive substances (TBARS) was used to assess peroxidation (Slater, 1984). A great variety of oxidized substances forms pink TBA complexes, such as malonaldehyde, oxidized sugars and amino acids (Esterbauer and Cheeseman, 1990), and all these compounds can be found in nuclear fractions submitted to an oxidative stress. After addition of 2 ml of 0.67% (w/v) TBA, 15% (w/v) trichloroacetic acid in 0.25 M HCl, samples were boiled in capped tubes for 10 minutes and cooled on fresh water. The reaction mixture was centrifuged at 1,500 ***g*** for 10 minutes. The optical density was measured at 535 nm against a blank without TBA.

Mitochondrial isolation

Mitochondria were isolated from brains of adult rats by differential centrifugation. Brains were homogenized using a ground-glass pestle in 0.2 M mannitol, 75 mM sucrose, 1 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid (EGTA), 0.05% (w/v) bovine serum albumin (BSA), 1 mM Tris buffer (pH 7.4). Homogenates were incubated in the absence (controls) or in the presence of 1 mM 3-methylcatechol for 0, 15 or 30 minutes at 37 °C before the centrifugation. Two independent experiments were done. Therefore, whole cells, nuclei, cytoskeletons and plasma membranes were removed by centrifugation at 550 ***g*** for 10 minutes at 4 °C, followed by

centrifugation of the supernatant at 7,100 ***g*** for 10 minutes at 4 °C. The mitochondrial pellet was resuspended in the same buffer and washed twice at 6,400 **g** for 10 minutes at 4 °C. Finally, mitochondria were resuspended in 1 ml of the same isolation buffer. Protein determinations were performed as described for nuclear fractions.

Oxygen electrode measurements

Oxygen consumption was carried out at 37 °C in a closed chamber containing a Clark type oxygen electrode connected to a monitor. Isolated mitochondria were suspended in 3 ml of 10 mM KCl, 0.2 mM EDTA, 0.25 M mannitol, 0.025% (w/v) BSA, 10 mM Tris, 5 mM phosphate buffer (pH 7.4) at a final concentration of 0.4 mg protein/ml. Sodium succinate was added to a final concentration of 10 mM in order to induce mitochondrial basal respiration (state 2). Oxygen uptake in resting conditions was monitored for 12 minutes. Four assays were analyzed for each mitochondrial fraction.

Cell cultures

Cultures of isolated cortical astrocytes were prepared as previously described (Booher and Sensenbrenner, 1972). Briefly, astrocytes were prepared from the neocortex of 1-day-old Wistar rat brains, maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 IU/ml), and streptomycin (100 □g/ml). Cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 ºC, and the culture medium was replaced three times a week. Cultures were used after they had attained confluence. At experiment, cells were seeded at a density of 7.8×10^4 cells/cm^2 in a 96-well plate and treated 72 hours later.

Treatment of cells with 3-methylcatechol

Sterile dilutions of 3MC were made up in 0.01 M HCl. Logarithm dilutions in the range of 60 to 3,000 μM were used to examine the cytotoxic effect of 3MC towards astrocytes and to determine the EC_{50}, the concentration of 3MC that killed 50% of cells. Eight replicates for each dose were used per 96-well plate. Cultures were exposed to 3MC for 72 hours.

Cell viability and catechol autoxidation

Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described previously (Hansen et al., 1989). In brief, this colorimetric assay measures the reduction of MTT by mitochondrial succinate dehydrogenase in intact cells. Because reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of cell viability. The 3MC oxidation in the medium was monitored spectrophotometrically at 405 nm based on quinones and melanin-like pigment formation (Lai and Yu, 1997).

Statistical Analysis

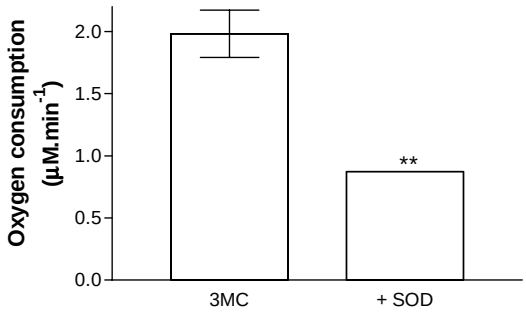
Results are expressed as the mean ± SD for each group and Student's *t* test was used to evaluate statistical differences. Results are expressed as

median, 25th, and 75th percentiles when normality test fails and in this case, Mann-Whitney U test was used to assess statistical differences. Differences were considered to be statistically significant at $P < 0.05$. A nonlinear regression was performed to fit concentration-response curves. Statistical comparisons between many groups were performed by analysis of variance (ANOVA) with the post hoc Student-Newman-Keuls test. Data were analyzed by the Kruskal-Wallis non-parametric ANOVA with the post hoc Dunn's method when the normality test failed.

RESULTS

The oxygen consumption during autoxidation of 1 mM 3-methylcatechol was estimated quantitatively. The rate of oxygen consumption was 1.98 ± 0.19 $\mu M.min^{-1}$ (mean ± SD, n = 15). From Fig. 1A it can be seen that the addition of 17U SOD significantly inhibited oxygen consumption by 56%. The formation of quinones during the autoxidation of 3MC increases the optical density (OD) at 320 nm. The rate of the increase in OD was 0.0021 ± 0.0002 min^{-1} (mean ± SD, n = 5). The rate of quinones formation was significantly decreased by 16% in the presence of 17 U SOD (Fig. 1B). Altogether, these data suggest that 3MC spontaneously reacts with oxygen in physiological conditions producing superoxide and reactive quinones. Thus, the possibility that the exposure of cells to 3MC could produce an oxidative damage was considered.

A



B

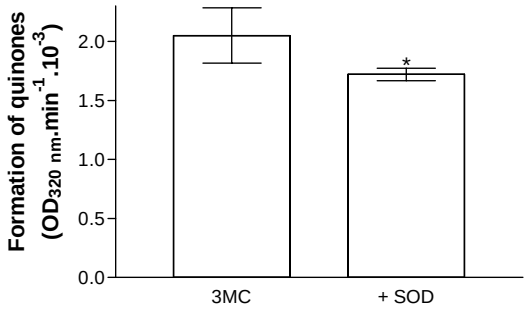


Figure 1 – (A) Oxygen consumption during autoxidation of 1 mM 3-methylcatechol in 1 mM HCl, 50 mM phosphate buffer (pH 7.4) at 37 °C. 3-Methylcatechol was tested alone (3MC, n = 15) or in the presence of 17 U superoxide dismutase (+SOD, n = 3). Each value represents the mean ± SD, (**), $p < 0.0001$. **(B)** Formation of quinones during autoxidation of 1 mM 3-methylcatechol in 1 mM HCl, 50 mM phosphate buffer (pH 7.4). 3-Methylcatechol was tested alone (3MC, n = 5) or in the presence of 17 U superoxide dismutase (+SOD, n = 4). Each value represents the mean ± SD, (*), $p < 0.05$.

We examined the effects of 3MC on the peroxidation of biomolecules found in nuclear fractions of rat brain cells. The formation of TBARS significantly increased compared with the spontaneous peroxidation in negative controls (58.4%, $P < 0.001$; Fig. 2A). The peroxidation in positive controls treated with 5 µM Fe_2SO_4 and 500 µM ascorbate for17 hours

increased by about 153.2% with respect to negative control values (P < 0.02; Fig. 2B). These data show that a 17-hour exposure of nuclear fractions to 1 mM 3-methylcatechol leads to an oxidative stress.

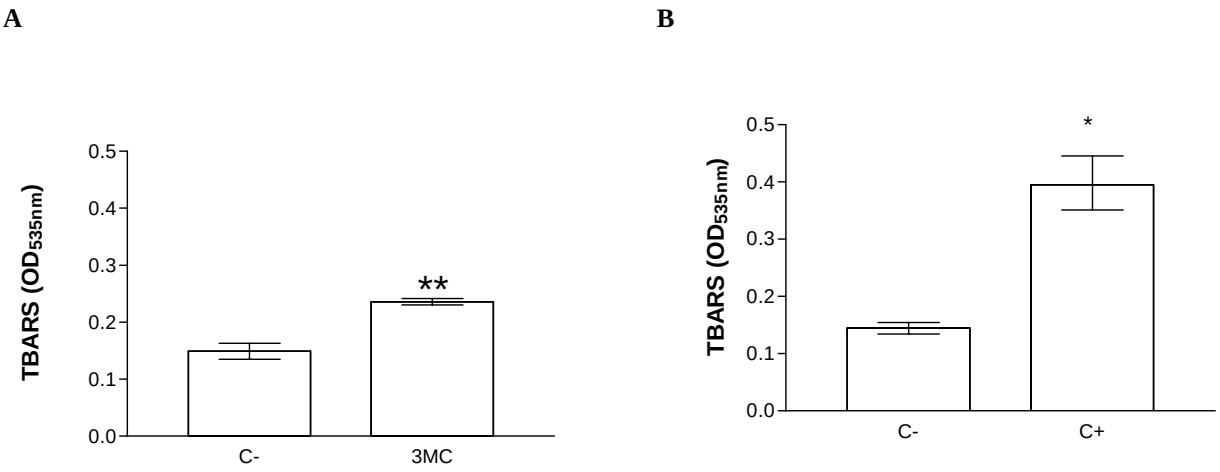


Figure 2 – (A) Mean ± SD of TBARS assay measured in rat brain nuclear fractions (1 mg protein/ml) incubated in the absence (C-) or in the presence (3MC) of 1 mM 3-methylcatechol at room temperature for 17 hours. Student's *t* test was used for comparison, (** $p < 0.001$), (n = 3). **(B)** Median, 25th, and 75th percentiles of TBARS assay measured in rat brain nuclear fractions (1 mg protein/ml) incubated in the absence (C-) or in the presence of 5 μM Fe_2SO_4 and 0.5 mM ascorbate (C+) at room temperature for 17 hours. Mann-Whitney U test was used for comparison, (* $p < 0.02$), (n = 3).

The rate of oxygen consumption in mitochondria isolated from rat brain homogenates during state 2 $FADH_2$-linked respiration was 2.5 ± 0.3 nmol.min^{-1}.mg protein^{-1}. This rate presented a 40% decrease when mitochondria were isolated after the addition of 3MC (1 mM) to brain homogenates immediately prior to centrifugation (Fig. 3). In another group, mitochondria were isolated from brain homogenates after incubation at 37 °C for 15 minutes. The rate of oxygen consumption during state 2 $FADH_2$-linked respiration in this group was 3.3 ± 0.2 nmol.min^{-1}.mg protein^{-1}. We demonstrated that this rate decreased by 51% when mitochondrial fractions were obtained after the addition of 3MC (1mM) to brain homogenates and incubated at 37 °C for 15 minutes prior to centrifugation. In the same resting conditions, the mean rate of $FADH_2$-linked oxygen consumption was 2.8 ± 0.1 nmol.min^{-1}.mg protein^{-1} when mitochondrial fractions were obtained after an incubation of brain homogenates at 37 °C for 30 minutes. Thus, mitochondrial basal respiration was inhibited by 51% when 1 mM 3-methylcatechol was added to brain homogenates and incubated at 37 °C for 30 minutes prior to isolation of mitochondria. Presumably 3MC inhibits electron flow between Complex II and Complex IV through pathways mediated by phenolic or quinone group substituents on the phenyl ring.

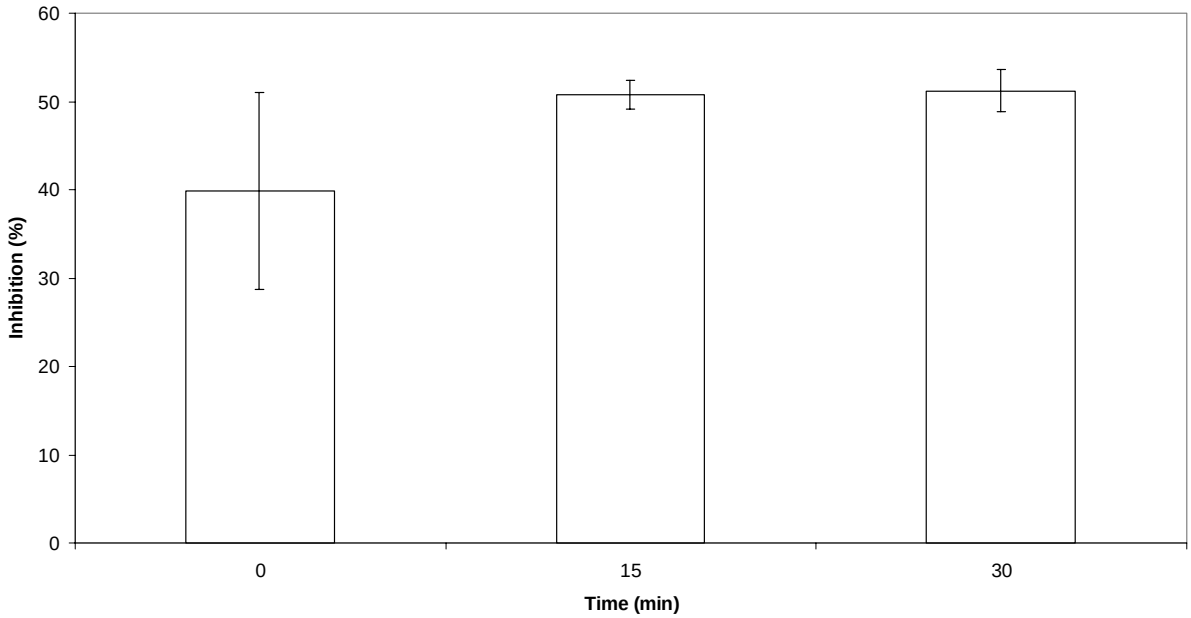


Figure 3 – Mean ± SD of 3MC-induced inhibition of state 2 $FADH_2$-linked mitochondrial respiration relative to controls. Rat brain homogenates were incubated in the absence (controls) or in the presence of 1 mM 3-methylcatechol for 0, 15 or 30 minutes at 37 °C before the centrifugation to obtain mitochondria. Two independent experiments were done. Oxygen uptake in resting conditions was monitored for 12 minutes after the addition of 10 mM sodium succinate. Four assays were analyzed for each independent experiment.

3-Methylcatechol is probably cytotoxic towards cells from the central nervous system since peroxidation of biomolecules and inhibition of state 2 $FADH_2$-linked mitochondrial respiration were induced by this compound in vitro. The formation of quinones and cell viability was then assayed in cultures of isolated primary astrocytes to determine the cytotoxicity of 3MC. Cell viability was measured by the use of MTT, which is a substrate for mitochondrial succinate dehydrogenase in intact cells. 3-Methycatechol oxidation led to a concentration-dependent formation of quinones in the medium of rat astrocyte cultures treated with this molecule for 72 hours (Fig. 4). Optical densities measured at 405 nm in the medium of control cells that were not treated with 3MC were significant different from all other groups 0.012 ± 0.005 (mean ± SD; n = 7). The formation of quinones well fitted ($R^2 = 0.981$) to equation [1]:

$$OD = 0.07 + \{0.10/[1 + 10^{(5.34 - 2.33 \log [3MC])}]\} \quad \textbf{[1]}$$

where OD corresponds to optical density at 405 nm and [3MC] is the 3-methylcatechol concentration.

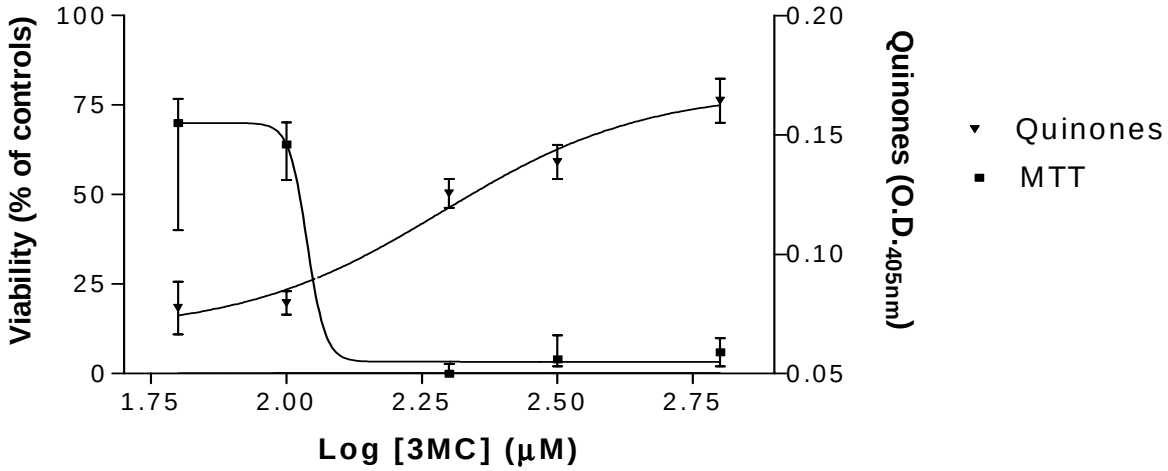


Figure 4 - Concentration-response curves for quinone formation and 3-methylcatechol-induced cytotoxicity in rat astrocyte cultures after 72 hours. The formation of quinones during the autoxidation of 3MC was measured by colorimetry at 405 nm (▼). Data for the autoxidation are represented by the mean ± SD, n = 8. The mean of optical densities obtained for the control group was 0.012. 3-Methylcatechol autoxidation led to a significant formation of quinones at all concentrations tested ($P < 0.05$). Statistical significance was analyzed using one-way ANOVA with Student-Newman-Keuls test. 3-Methylcatechol also alters the viability of these cells (■). Viable cells were quantified colorimetrically by the MTT assay (see Materials and Methods) following 72 hours in the presence of 3MC. Data were normalized to the values measured under control conditions (median of optical densities at 560 nm = 0.5). 3-Methylcatechol caused a significant reduction of cell viability at concentrations above 100 µM. Results are shown as medians ± 25^{th} and 75^{th} percentiles, n = 8. Statistical significance was analyzed using the Kruskal-Wallis one-way ANOVA with Dunn's method.

Whereas the level of quinones increased with the concentration of catechol, an induction in cell death was detected (Fig. 4). Control cells reduced MTT and the median of the OD at 560 nm calculated for this group (median, 0.500; 25^{th} percentile, 0.480; 75^{th} percentile, 0,517; n = 7) was considered as 100% viability. The cell viability of groups treated with concentrations of 3MC above 100 µM significantly differed from the control one. We calculated the EC_{50} of 3MC toward rat astrocytes that corresponded to 107 µM after 72 hours. Equation [2] represents the non-linear regression calculated from these data ($R^2 = 0.9962$):

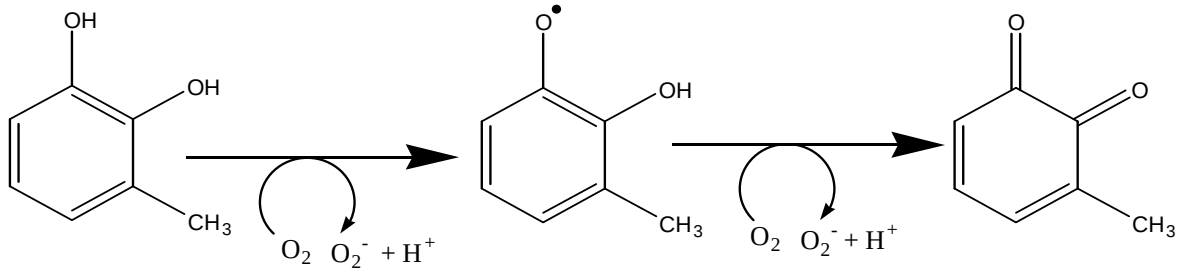
$$V = 3.33 + \{66.67 / [1 + 10^{(-46.40 + 22.70 \log [3MC])}]\} \quad \textbf{[2]}$$

where V is the cell viability normalized to the values measured under control conditions and [3MC] corresponds to 3-methylcatechol concentration.

DISCUSSION

Deliberate or involuntary inhalation of organic solvents causes toxic effects and behavioral changes. Thinner is a neurotoxic mixture containing 60-70% toluene, which induced an increase of lipid peroxidation products in hippocampus, cerebellum and cortex of rats chronically exposed to this solvent (Baydas et al., 2005). Toluene is a solvent that permeabilizes mitochondria (Giulivi et al., 1998).

This compound induced the formation of ROS in rat brain synaptosome fractions (Myhre and Fonnum, 2001). This molecule at concentrations up to 178 μg/ml did not present cytotoxic effects towards CD3-CD28-stimulated human peripheral blood mononuclear cells (Wichmann et al., 2005). However, 1 mM toluene increased caspase-9 activity in LLC-PK1 immortalized renal proximal tubular epithelial cells derived from pig treated for 96 hours (Al-Ghamdi et al., 2004). Toluene was also toxic towards primary cultured hippocampus neurons at concentrations above 3 mM after 24 hours (Yan et al., 2004).



3-Methylcatechol **3-Methyl-*o*-benzosemiquinone** **3-methyl-*o*-benzoquinone**

Scheme 1 – Oxidation of 3-methylcatechol

In this work we studied the toxicity of 3MC, which is a metabolite of toluene. The measurement of oxygen consumption during the autoxidation of 1 mM 3-methylcatechol in 1 mM HCl, 50 mM phosphate buffer (pH 7.4) showed that 1.98 μM oxygen reacts with 3MC per minute leading to the formation of superoxide. 3-Methyl-*o*-benzosemiquinone and 3-methyl-*o*-benzoquinone, which absorb light at 320 nm, are also formed. These results agree with data obtained during the electrochemical oxidation of 3MC (Fakhari et al., 2005). These observations allow us to propose the pathway in Scheme 1 for the reaction of 3MC with oxygen.

We showed for the first time that 3MC induces the peroxidation of biomolecules in rat brain nuclear fractions. 4-Methylcatechol at concentrations up to 100 μM did not induce lipid peroxidation of rat liver microsomes (Boots et al., 2002), however the formation of quinones induced a thiol arylation of proteins, which inactivated some enzymes. Several catechols derived from arenes with a heterocyclic group at low concentrations inhibited the lipid peroxidation induced by free radicals in rat brain homogenates (Misawa et al., 2005), but these authors did not test the toxicity of these compounds at higher concentrations.

In the present study, we also showed for the first time that 3MC significantly inhibited state 2 $FADH_2$-linked respiration of rat brain mitochondria. Our study shows that 3MC has the potential to interfere with oxidative energy metabolism in vitro. It has been reported that other catechols also interfere in mitochondrial function. In a previous work (Barreto et al., 2005), we found that 1,2-dihydroxybenzene (catechol) inhibited state 2 $FADH_2$-linked respiration of rat liver mitochondria. Nordihydroguaiaretic acid, which is a natural catechol considered as an antioxidant, induced the membrane depolarization of mitochondria in isolated porcine coronary arterial smooth muscle cells (Yamamura et al., 2002). Rats treated with another catechol, tolcapone, which is a catechol-*O*-methyl transferase inhibitor, presented a decreased mitochondrial ATP synthesis in liver (Haasio et al., 2002). This molecule also decreased both the glutamate and succinate dependent ATP synthesis on cultured human neuroblastoma cells (Korlipara et al., 2004). Quinones binding to cysteinyl groups in proteins, and since mitochondrial proteins are rich in sulfhydryl moieties this may be the reason for the inhibition of mitochondrial complexes and the opening of the mitochondrial permeability transition pore induced by oxidized catechols (Kostrzewa et al., 2002).

As 3MC is able to produce superoxide and reactive quinones, to induce peroxidation of biomolecules and to inhibit $FADH_2$-linked respiration, these processes could account for cytotoxic effects. We demonstrated in this study that cell viability decreased in cultures of isolated rat cortical astrocytes after treatment with 3MC. The decrease in cell viability was accompanied by the production of quinones in the culture medium. The EC_{50} was 107 μM after 72 hours. These data correlate with the observed cytotoxicity of 3MC towards bluegill sunfish BF-2 fibroblast cells (Shen et al., 2000)

indicating that this metabolite of toluene represents a hazardous environmental pollutant. Other catechols are also cytotoxic compounds. In a previous study (Pereira et al., 2004) we demonstrated that catechol was cytotoxic towards human glioblastoma cells via the production of superoxide and reactive quinones. Catechol at 230 μM killed 50% of these cells after 72 hours. In another work (El-Bachá et al., 2001), we studied the neurotoxicity of apomorphine, which is a catechol that is a potent dopamine agonist, and showed that the toxicity of this drug is also related to the autoxidation and formation of ROS and quinones. Apomorphine at 200 μM killed 50% of glial C6 cells after 48 hours (El-Bachá et al., 2001).

The toxicity of 3MC, one of the major aromatic intermediates of aerobic transformation of toluene in the environment, was evaluated in organelles and cells of rat brains. This toluene metabolite can elicit peroxidation of biomolecules, inhibition of state 2 $FADH_2$-linked mitochondrial respiration and death of astrocytes. The results of the study indicate a direct relationship between the cytotoxicity and the oxidation of 3MC. Since the cytotoxic effects induced by 3MC involves an oxidative process, this model may be technologically used for screening the protective and antioxidant activity of biomolecules obtained from natural products.

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RESUMO

O 3-metilcatecol (3MC) é um metabólito do tolueno. Para esclarecer se o 3MC seria tóxico para o sistema nervoso central, examinou-se seus efeitos sobre a peroxidação de biomoléculas em frações nucleares e a respiração mitocondrial em organelas obtidas de cérebros de ratos. Também se testou a citotoxicidade para astrócitos primários de ratos. O 3MC a 1mM oxida-se consumindo oxigênio a uma taxa de 1,98 ± 0,19 $\mu M.min^{-1}$, formando quinonas reativas. Nessa mesma concentração o 3MC peroxidou biomoléculas nas frações nucleares. Esse composto também inibiu o estado 2 da respiração mitocondrial associada ao $FADH_2$. Além disso, o 3MC também se oxida em meio de cultura levando à formação de quinonas. Esse metabólito do tolueno foi citotóxico para astrócitos de ratos. A concentração que matou 50% das células após 72 horas foi 107 μM. Os resultados desse estudo indicam uma relação direta entre a citotoxicidade e a oxidação do 3MC.

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3.4 Artigo IV – DNA damage in rat brain cells treated with the prooxidant catechol

Tomando como referência a metodologia empregada por outros pesquisadores para realizar os estudos que enfatizam a genotoxicidade e mutagenicidade de compostos aromáticos, testamos a hipótese do catecol (pirocatecol) induzir uma lesão de DNA em um modelo de células cerebrais de ratos. Este estudo foi totalmente viabilizado no Laboratório de Biologia Molecular e Genômica (LBMG) da Universidade Federal do Rio Grande do Norte, onde tentou-se reproduzir o modelo e adequá-lo às expectativas e necessidades.

Os resultados demonstram um aumento na ocorrência de cometas de níveis 2 a 4 de forma dose dependente, sugerindo que as espécies reativas de oxigênio geradas pela oxidação do catecol induzem danos ao DNA, o que pode também estar relacionado com a citotoxicidade deste composto.

Este manuscrito será submetido para a apreciação dos editores do periódico **Mutation Research**.

A cópia do artigo, o qual está em fase final de revisão, encontra-se nas próximas páginas (81 a 97).

DNA damage in rat brain cells treated with the prooxidant catechol

George Emílio Sampaio Barreto[1,2], Leonam Gomes Coutinho[1], Ramon dos Santos El-Bachá[2], Lisandro Diego Alvarez Giraldez[2], Silvia Lima Costa[2], Lucymara Fassarella Agnez Lima[3] and Eryvaldo Sócrates Tabosa do Egito [4]*

[1]Programa de Pós-Graduação em Ciências da Saúde (PPGCSA) – Universidade Federal do Rio Grande do Norte (UFRN) – Natal/Brasil, [2] Laboratório de Neuroquímica e Biologia Celular (LabNq) – UFBA, Salvador/Brasil,[3] Laboratório de Biologia Molecular e Genômica (LBMG) – UFRN, [4] Laboratório de Sistemas Dispersos (LASID) – UFRN, Natal/Brasil.

Abstract

Catechol is a genotoxic agent assumed to induce DNA damage via the oxidative pathway. To estimate the catechol toxicity, DNA damage was determined in rat brain cells incubated at up to 20 minutes with increasing concentrations of catechol. A comet assay was carried out in order to evaluate the DNA damage, and the cells were classified as 0-4 damage level. Tail lenght was measured in order to quantitate the harm extension. The results suggest that rat brain cells are sensitive towards catechol-induced DNA damage in a dose- and time-dependent manner, as observed by increasing the tail lenght of the comet, when the cells were subjected to alkaline single cell electrophoresis. When the cells were treated in the presence of 10 mM catechol , the damage was considerably higher when compared to the control group with HCl, representing 200% higher, and even so to hydrogen peroxide control group (positive control) which was about 150% higher. This effect was found to be dose related. The remarkable aspect of this compound was its ability to induce an inhibition of state 2 $FADH_2$-linked mitochondrial respiration, a cytotoxic effect towards human glioblastoma, moreover, in the present study, an oxidative DNA damage was observed, probably due to semiquinones and reactive quinones generation.

[4] Correspondence: +55 (84) 3215-4355 – email: sócrates@ufrnet.br

Introduction

Catechol (1,2-dihydroxybenzene), a naturally occurring chemical in cigarette smoke, is used as a reagent for photography, rubber and plastic production, and in the pharmaceutical industry (1). Its is readily absorbed from the gastrointestinal tract, and through vasoconstriction, degenerates the renal tubes in the kidneys, diminishes liver function and accumulates in the bone marrow (2).

Catechol is also a major metabolite of benzene, which is known to cause myelotoxicity and leukaemia in humans (3). The molecular mechanisms of mutagenicity of phenolic compounds in mammalian cells are unknown. Although the genotoxicity of catechol has been extensively examined, the mechanisms remain to be determined (1, 4-10).

Several studies have demonstrated that catechol can cause DNA damage (9,11) including DNA strand breaks (12). It is known that catechol auto-oxidises in aqueous physiological pH conditions increasing active oxygen species such as the superoxide anion (13), suggesting that DNA lesions mechanisms could be mediated by the production of radical species. Indeed, ROS-initiated DNA damage includes oxidized bases, abasic sites, DNA-DNA in strand adducts and DNA protein crosslinks. Catechol binds covalently to cellular molecules, proteins and DNA in tissues. This binding is implicated in mechanisms of toxicity (inhibiting cell replication) and carcionogenicity (inititation of leukemia, 14). Therefore, it plays a critical role in cell death and carcinogenesis (15), probably acting as a mutagen via an indirect mechanism, leading to oxidative DNA damage through the formation of hydroxyl radicals via hydrogen peroxide (11).

Numerous studies over the years have described and evaluated the adverse health effects associated with catechol exposure (16). Recently, we demonstrated that catechol is able to induce an inhibition of state 2 $FADH_2$-linked mitochondrial respiration, probably due to semiquinones and reactive quinones generation and consequently, ROS formation (17). We previously found that catechol was cytotoxic towards human glioblastoma cells via the production of superoxide and reactive quinones (18). It is worthwile noting that the neurotoxicity of apomorphine, a catechol that is a potent dopamine agonist is also related to the autoxidation and formation of ROS and quinones (19).

From these data, we hypotethize that the oxidation of catechol could lead to deleterious oxidative effects in DNA, since this compound is toxic towards cells and its compartments. Current concern is focused on the effects of long-term occupational exposure and environmental exposure to catechol (20).

In order to identify and quantify DNA damage, comet assay or single cell gell electrophoresis (SCGE) has been assigned and extensively used. This assay has been widely used in order to detect strand breaks, alkali-labile sites, DNA crosslinking and incomplete excision repair sites. The technique has proven to be a very good sensitive method and a useful tool for the detection of genetic damage at the individual cell (21-22). A significant advantage of the SCGE assay is its applicability to any eucaryotic organism and cell type. Since the assay is also inexpensive and gives results within a few hours, it is appropriate for environmental monitoring.

Since the mechanism of DNA damage and consequently its carcinogenicity has not been clarified in the central nervous system, probably due to the lack of information in this model, we were interested in studying the DNA rat brain cells damage treated with the pro-oxidant catechol by using the comet assay.

Material and Methods

Animals

Adult Wistar rats weighing 250-350g were obtained from the Department of Experimental Surgery of the Health Sciences Centre of the Federal University of Rio Grande do Norte (Natal, RN, Brazil). All experimental protocols were conducted according to the regulations suggested by the Federal University of Rio Grande do Norte Ethical Committee.

Comet Assay

Brain cells sample

Animals were sacrificed by decapitation and the forebrain was collected, placed in 5 ml of cold phosphate-buffered saline (pH 7.4) and minced into fine pieces in order to obtain a cellular suspension. Cell treatment was done in a time and concentration-dependent manner (1

mM; 5 mM and 10 mM incubated at 37 ºC for 0, 10 and 20 minutes, respectively). 1 ml of the cell suspension was treated with catechol, and about 3 µl of this sample were mixed with 75 µl of 0.5% low melting point agarose (LMA) (Gibco – BRL) for embedding on slides.

Slide Preparation

The comet assay was performed according to Singh et al. (1988) with minor modifications. Roughened microscope slides were dipped briefly into 1.0% (60 ºC) normal melting agarose (NMA) prepared in phosphate-buffered saline (PBS). The slides were dried overnight at room temperature and then stored at 4 ºC until use. Microscopic slides were each covered with 300µl of 1% NMA at about 60°C in Ca^{2+}- and Mg^{2+}- free PBS. They were immediately covered with a large coverslip and kept at refrigerator temperature for 10 min to allow agarose to solidify. This layer was used to promote the attachment of the second layer of 0.5% LMA. Treated cells (about 3 µl) were suspended with 75 µl of 0.5% LMA. After gently removing the coverslip, the cell suspension was rapidly pipetted onto the first agarose layer, spread out with a coverslip and maintained at 18°C for 10 min to solidify. After removal of the coverslip, the slides were immersed in a cold lysing solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% sodium sarcosinate, pH 10) with 1% Triton X-100 and 10% DMSO added just before use, for at least 1 h at 4°C.

Electrophoresis

The slides were removed from the lysing solution, drained and placed in a horizontal gel electrophoresis tank near the anode. The tank was filled with cold electrophoresis solution (200 mM Na_2EDTA and 10 N NaOH, pH 13) to a level approximately 0.25 cm above the slides. Before electrophoresis, the slides were left in the solution for 20 min to allow the unwinding of the DNA and the expression of alkali-labile damage. Electrophoresis was conducted at 1.6 V/cm for 20 min (300 mA) in ice immersion.

The slides from all subjects were electrophoresed on the same day. All these steps were conducted under dimmed light. After this process, the slides were washed 3 times with a neutralization buffer (0.4M Tris, pH 7.5) to neutralize the excess alkali and they were allowed to sit for 5 min.

Staining

Each slide was stained with 50 µl ethidium bromide (EtBr-20 µg/ml), covered with coverslip and placed in a humidified air-tight container to prevent drying of the gel and analyzed within 3-4 h.

Slide Scoring

One hundred cells per subject were analyzed at 400x magnification, under a fluorescence microscope equipped with an excitation filter of 546 nm and a barrier filter of 590 nm. Comets are formed upon the principle of releasing damaged DNA from the core of the nucleus during electrophoresis. At low damage levels, streching of attached strands of DNA is likely to occur. With increasnig numbers of breaks, DNA pieces freely migrate into the tail of the comet.

The cells were classified by eye into 4 categories on the basis of the extent of migration, 0 (undamaged), 1 (very low migration), 2 (low migration), 3 (medium to high damage) and 4 (high migration). Tail lenght (in micrometers, measured from the edge of the comet head – 1.3µm/bar) was used as a parameter to identify the entension of DNA damage.

Statistical Analysis

Results are expressed as median, 25^{th}, and 75^{th} percentiles when normality test fails. Differences were considered to be statistically significant at $P < 0.05$. Statistical comparisons was analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks.

Results

The results obtained indicate the ability of catechol to induce DNA damage in rat brain cells exposed to increasing times of incubation. Firstly, we evaluated the probable nucleic acid damage in rat brain cells exposed to 0.01M HCl, 10mM H_2O_2 (hydrogen peroxide) and 1mM catechol (control groups): Fig. 1 presents the extent of DNA damage in three different controls – HCl, H_2O_2 as a positive control, and catechol (Cat) of rat brain cells. Note that

there was no significant statistically difference between H_2O_2 and Cat. However, both groups are 50% greater when matched to control with HCl (* $P < 0.05$). Catechol has the capacity to auto-oxidize in an aqueous solution (1) and hydrogen peroxide is considered as a subproduct of its metabolization. Then, It is assumed that both compounds are able to produce some damage in DNA.

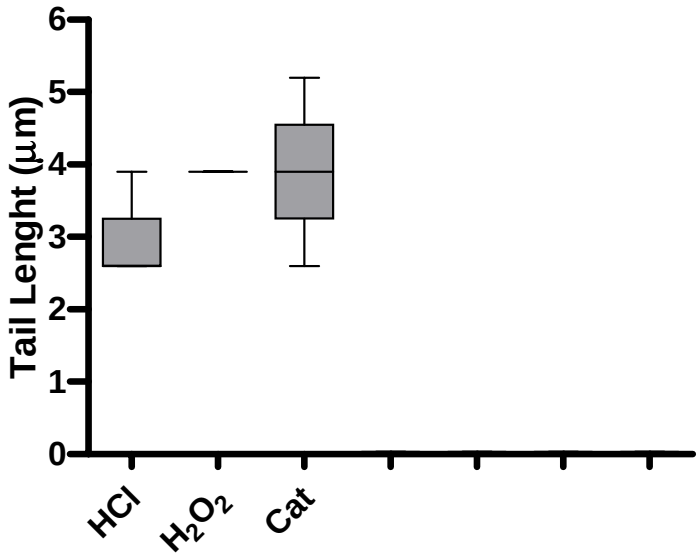


Fig. 1. Median, 25th, and 75th percentiles of rat brain cells incubated with HCl 0.01M, H_2O_2 10 mM and catechol 1mM (Cat) expressed as tail lenght. Two independent experiments were performed and 50 cells per slide were quantified. There is no statistically difference between H_2O_2 and Cat. Statistical significance was analyzed using the Kruskal-Wallis One Way Analysis of Variance on Ranks (* $P < 0.05$).
* $P < 0.05$ compared with control (HCl).

Despite of the results shown in Fig. 1, we also evaluated in Fig.2 the distribution of tail lenght in rat brain cells exposed to catechol at different concentrations incubated at T= 0 minute (37º C) when compared to control with HCl. Catechol at 1mM and 5mM did not show statitiscal difference, however, they were significant about 33.3% higher tail lenght when matched to control group (* $P < 0.05$). Catechol at 10mM represents a value of tail lenght about 100% greater DNA damage than the reference value, and 25% higher than catechol at 1mM.

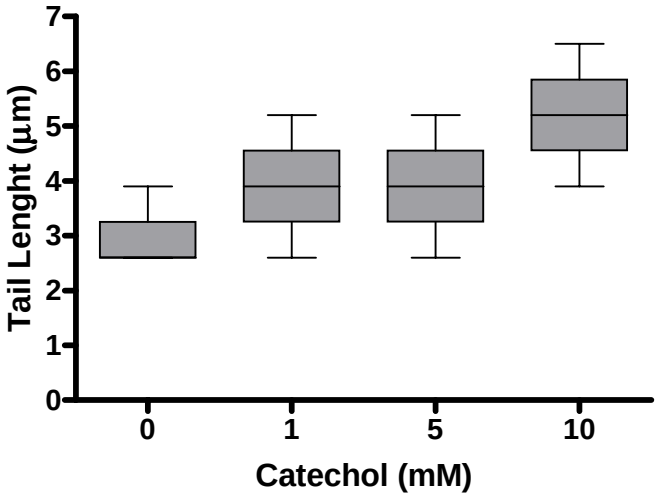
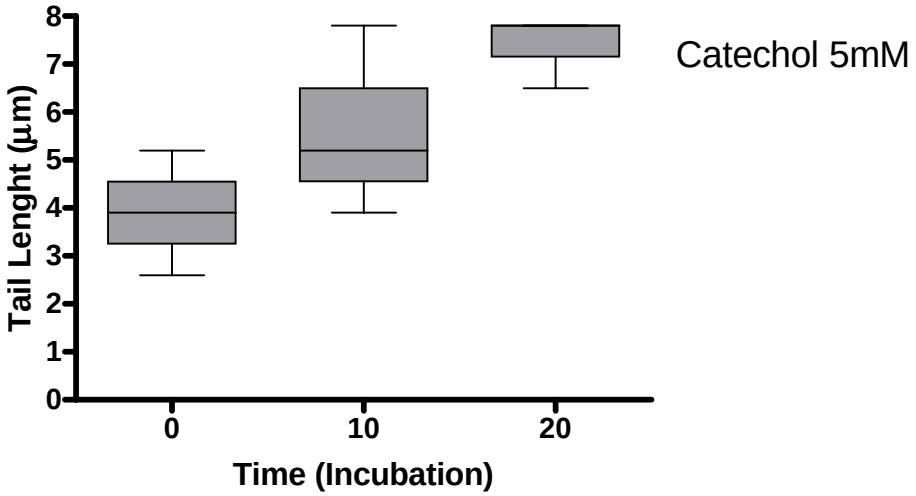
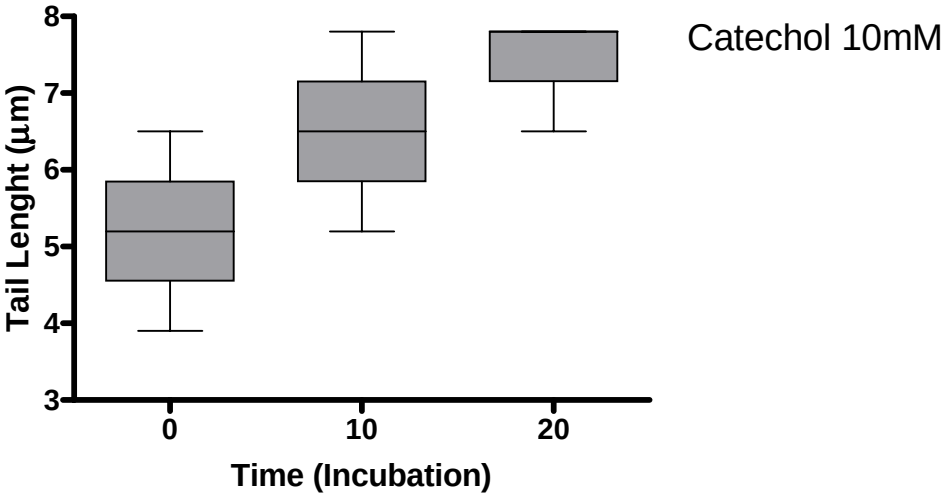


Fig.2 - Median, 25th, and 75th percentiles of DNA damage in rat brain cells exposed to catechol at 0mM, 1mM, 5 mM and 10mM with T=0 incubation. 150 cells were scored and compared with control (HCl). Statistical significance was analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks (* $P < 0.05$).

In Fig. 3A, we investigated the distribution of tail lenght (Median, 25th, and 75th percentiles) of rat brain cells exposed to 5mM catechol at 0, 10 and 20 minutes of incubation (37º C). There is a statistically significant difference (* $P < 0.05$) among catechol-incubated rat brain cells at 0 minutes, 10 minutes and 20 minutes. It is likely to be dose-concentration dependent since only the time-exposition has varied over the constant and known catechol concentration. Incubation with catechol at 20 minutes is about 100% greater than catechol at 0 minutes and 33.3% when matched to catechol-incubation at 10 minutes. 1mM catechol 10 minutes is different about 33,3% of that one at 0 minutes. From this data, we can suggest that exposition time is stricly related to DNA damage; In Fig. 3B demonstrates the distribution of tail lenght of rat brain cells exposed to 10 mM catechol at 0, 10 and 20 minutes of incubation (37º C). There is a statistically significant difference (* $P < 0.05$) among catechol-incubated rat brain cells at 10 minutes (20% greater DNA damage) and at 20 (33.3% higher) when compared to 1mM catechol at 0 minutes.



(A)

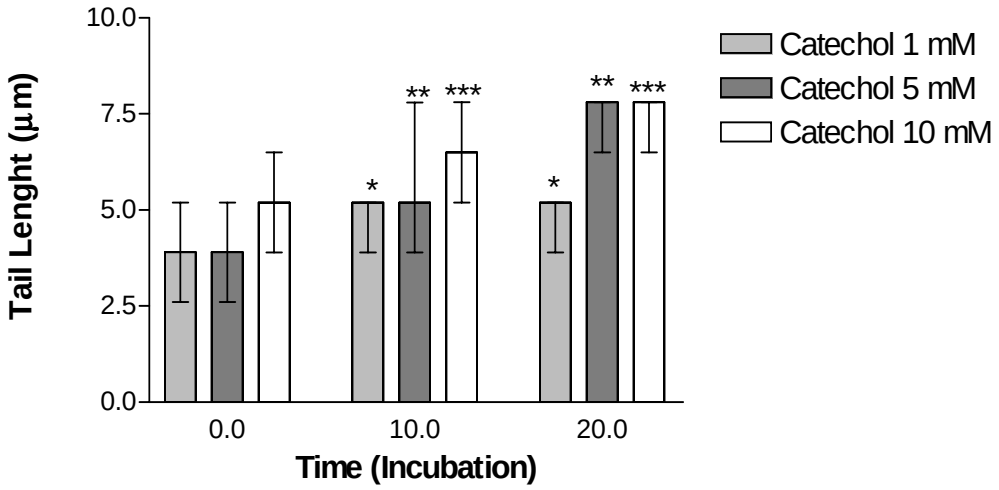


(B)

Fig.3. **(A)** DNA damage in rat brain cells exposed to 5mM catechol incubated for 0, 10 and 20 minutes at 37º C. Values represent the medians, 25th and 75th percentiles of two independent experiments, in which 100 cells per incubation time were scored. **(B)** DNA damage in rat brain cells exposed to 10mM catechol incubated for 0, 10 and 20 minutes (37º C). 150 cells were quantified and matched with control (HCl). Statistical significance was analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks.

***** $P < 0.05$ compared with T= 0 incubation

≠ $P < 0.05$ compared with T= 10 minutes incubation



* P < 0.05 vs. 1 mM, 0 min
** P < 0.05 vs. 5 mM, 0 min
*** P < 0.05 vs. 10 mM, 0 min

Fig. 4- Concentration and time-incubated rat brain cells expressed as median, 25th and 75th percentiles of two independent experiments. Two independent experiments were performed and 150 cells were analysed. Statistical significance was analyzed using Kruskal-Wallis One Way Analysis of Variance on Ranks (* P < 0.05).

In a way to demonstrate and compare the results in a concentration-time dependent manner, Figure 4. emphasizes DNA damage expressed as tail lenght (µm) in function of time-incubated rat brain cells exposed to 1, 5 and 10mM catechol. Tail migration of cells exposed to 1mM catechol increased by about 25% (incubated at 10 and 20 minutes) whether compared to cells incubated at 0 minutes. Furthermore, we evaluated the DNA damage in 5mM and 10mM catechol groups ; results have indicated a higher damage of cells exposed to 5mM catechol by about 25% (at 10 minutes) and 100% (at 20minutes) when matched to 0 minutes exposition time. Also, in the maximum concentration of catechol used in this study (10mM) damage was likely observed, representing 16.6% (at 10minutes) and 33,2% higher at 20 minutes with respect to catechol-exposition time at 0 minutes. These data suggest that increasing time of catechol-exposition was directly related to DNA damage in such dependent way.

Discussion

Some studies have used the comet assay as a useful method to detect DNA damage in many biomonitoring systems (23-28) Although single-cell-gel electrophoresis is a rapid and sensitive procedure for quantitating DNA lesions in mammalian cells, there are few, or none, studies that emphasize its use in the central nervous system exposed to aromatic hydrocarbons. Based on the lack of informations over the brain cell DNA damages, we investigated the ability of catechol to induce DNA damage in rat brain cells through the comet standard assay, since in previous studies it has demonstrated to be toxic to mitochondria (17) and glioblastoma cells (18) via generation of semiquinones and reactive quinones.

Catechols readily undergo auto-oxidation in an aqueous solution, under a physiological pH, to form semiquinones radicals and quinones, which are more reactive than catechols. The mechanisms most frequently cited to explain the toxicity of catechols are: (i) the generation of reactive oxygen species by redox reactions; (ii) DNA damage in the form of oxidative damage or DNA arylation; (iii) protein damage by sulfhydryl arylation or oxidation; and (iv) interference with electron transport in energy transducing membranes (1,29). Moreover, catechol releases iron from ferritin inducing lipid peroxidation in brain homogenates (30). When catechol is enzimatically oxidized or in the presence of oxygen and heavy metals, one electron is transferred to the molecular oxygen, and consequently superoxide (O_2^-) is formed with further reduction to hydrogen peroxide (H_2O_2) and hydroxyl radicals (·OH). Whereas the hydroxyl radical is supposed to induce a more general DNA damage (12), catechol was found to induce a rather site specific damage in thymine, especially at the 50-GTC-30 sequence in DNA fragments from the human C-Ha-ras-1 proto-oncogene (12). Catechol can act as pro-oxidant damaging macromolecules such as DNA and proteins and destroying membrane functioning due to their redox cycling activity (1).

Despite of the peroxide production, catechol is not mutagenic in *E.coli and Salmonella typhimurium*, when tested alone or in combination with cooper (31-32). The absence of mutagenicity is probably due to activated defence systems, as for example, an induction of catalase. However, exposure of Syrian hamster embryo (SHE) cells to catechol induces cell transformation, gene mutations, unscheduled DNA synthesis, chromosomal aberrations and

sister chromatid exchanges (33). Catechol alone does not cause oxidative DNA damage in bone marrow as shown by Kolachana et al. (34). In combination with phenol or hydroquinone (neither compound induces oxidative DNA damage alone), however, the 8-hydroxy-2´-deoxyguanisine (8-oxodG) level increases, which is indicative of oxidative DNA damage (34). Oikawa et al. (12) demonstrated that the content of 8-oxodG in HL-60 cells was increased by catechol, whereas the content of 8-oxodG in HP100 cells was not increased. DNA base damage in the form of 8-oxodG is a prominent indicator of oxidative stress and has been well-characterized as a premutagenic lesion in mammalian cells. Catechol exhibits inhibitory effects on the DNA synthesis in the mouse lymphoma cell line L5178YS. These effects have been explained by DNA damage caused by DNA alkylation or oxidative DNA damage (34). However, similar effects in human T lymphoblasts have been explained by enzyme inhibition.

It is well-known that catechol has strong promotion activity. Many investigations have indicated that catechol strongly enhances cancer development in rats and mice initiated with carcinogens, such as benzo[a]pyrene and N-methyl-N'-nitro-N-nitrosoguanidine, due to observations have suggested that some tumor promoters act to produce DNA damage mediated by reactive oxygen species (35-39). Oikawa et al. (12) have demonstrated that catechol could induce metal-dependent H_2O_2 generation and subsequent damage to DNA fragments obtained from the human p53 and p16 tumor suppressor genes. Catechol also inhibits the rate limiting step of DNA synthesis in human T lymphoblasts, probably by inhibiting the ribonucleotide reductase. Compounds with catechol moieties and a benzenic ring in their composition, such as catechol estrogens, L-DOPA, dopamine and α-methyl-DOPA can also cross-link proteins (40-43), for example, L-DOPA and dopamine cross-link neurofilaments. Thus, substances with the catecholic moieties can cause DNA damage *in vitro* or *in vivo*, upon activation, for example, by heavy metals, or by cellular metabolism and conjugation reactions. DNA damage is either due to DNA-adduct formation by the catechols or their reaction intermediates, or due to the formation of ROS causing an oxidation of DNA bases and/or DNA strand breaks.

Previous studies have shown that the autoxidation products of apomorphine (APO) and other catechols (e.g. dopamine) might lead to deleterious effects on neuronal cells and neural function (44-45), The cytotoxic effects of APO to cultured neurons have been shown to correlate to its autoxidation products. Lévay et al. (46) have shown that the

neurotransmitter dopamine which has a catechol nucleus and that is found as an endogenous compound in plants and mammals caused DNA damage in two different ways: DNA adducts are formed and oxidative DNA damage upon incubation with cooper occurs (46). Picada et al. (47) showed a significant increase in DNA damage index and damage frequency in mice brain tissue 1 and 3 h after treatment with 8-OASQ, an autoxidation product of APO. Indeed, It has been demonstrated that 8-OASQ displays a higher frameshit mutagenic activity, which stimulates DNA strand breaks, when compared to APO (48). At this same study, 8-OASQ displays biological effects through the usual redox reactions of quinones and semiquinones generating H_2O_2 or O_2^- in addition to quinone and semiquinone radicals (49). These reactions could promote an increased formation of hydroxyl radicals that are able to induce mostly single-strand breaks and various species of oxidized purines and pyrimidines (50-51).

The DNA damage caused by catechol containinng fraction of aqueous cigarette tar (ACT) has also been investigated for its DNA damaging activity in vitro experiments. Hydrogen peroxide, superoxide and hydroxyl radicals are produced by this fraction and cause oxidative DNA damage (52). This ACT also contains the tar radical, consisting of polymerized catechol, suggesting that catechol is involved in the production of the ROS. The tar radicals bind to DNA and DNA adducts are formed (53). DNA damaging can also be seen through the activity of catechols estrogens which play an important role in semiquinones and quinones generation (54-55).

In the present paper we have demonstrated that rat brain cells exposed to increasing concentrations of catechol have produced DNA damage in a time and concentration dependent manner. We suggest that this toxicity towards DNA is due to generation of semiquinone and reactive quinones, and yet reactive oxygens species play an important role in the oxidative damage.

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3.5 Artigo V - Oxidative stress, inhibition of mitochondrial respiration and glial fibrillary acidic protein (GFAP) expression are involved in catechol-induced cytotoxicity.

Este estudo segue a mesma metodologia e parte experimental dos artigos anteriores. No entanto, o pirocatecol foi testado, além do modelo mitocondrial, em culturas astrocitárias e frações nucleares cerebrais, com o intuito de avaliar a sua toxicidade no SNC e a habilidade de induzir peroxidação lipídica.

Neste manuscrito, nos detemos a relatar os resultados obtidos no modelo mitocondrial de cérebro de ratos. Por sua vez, o pirocatecol produziu uma inibição parcial da respiração mitocondrial, mais proeminente se comparado nas frações cerebrias, quando incubados por 0 e 15 minutos a 37ºC.

O referido artigo está em fase final de elaboração dos resultados, e será submetido para a apreciação dos editores do periódico **Journal of Cell Science**.

A cópia do artigo, contendo os resultados no modelo mitocondrial, encontra-se nas próximas páginas (99 a 103).

Oxidative stress, inhibition of mitochondrial respiration and glial fibrillary acidic protein (GFAP) expression are involved in catechol-induced cytotoxicity

George Emílio Sampaio Barreto[1] et al.

[1] *Programa de Pós-Graduação em Ciências da Saúde (PPGCSA); Universidade Federal do Rio Grande do Norte (UFRN), Natal –Rio Grande do Norte – Brasil.*

INTRODUCTION

There is an increasing interest to study the toxicity of catechols toward cells from the central nervous system (CNS) since it has been speculated that it is associated with neurodegenerative diseases. Advances in the understanding of catechol toxicity are mainly due to studies about Parkinson's disease (PD) because the cornerstone of the treatment is the dopamine replacement therapy, which applies its precursors or receptor agonists [1]. These molecules are catechols in a large range or are metabolized to this kind of substance that is potentially toxic mainly due to the generation of reactive oxygen species (ROS) and quinones during its oxidation. In spite of the ability of catechols to autoxidize, other factors may contribute to increase neurodegeneration like polymorphisms of catechol-*O*-methyltransferase (COMT) [2-3], mitochondrial dysfunction and the accumulation of misfolded proteins [4]. Dopamine replacement therapy is associated with the emergence of debilitating involuntary movement, it does not slow the progression of degeneration of dopamine neurons, and its efficacy is reduced over time. Astrocytes may play a role in the progression of PD because a subpopulation of these cells (Gomori astrocytes) exhibiting an endogenous peroxidase activity catalyzes dopamine and also catechol estrogen oxidation to potentially neurotoxic *o*-semiquinone radicals [5]. However, although catecholamines and analogues share 1,2-dihydroxybenzene (catechol) as a common group, little is known about the effects of this benzene metabolite in cells from the CNS.

MATERIALS AND METHODS

Mitochondrial isolation

Mitochondria were isolated from brains of adult rats by differential centrifugation. Brains were homogenized using a ground-glass pestle in 200 mM mannitol, 75 mM sucrose, 1 mM EGTA, 0.05% (w/v) BSA, 1 mM Tris buffer (pH 7.4). Homogenates were incubated in the absence (controls) or in the presence of 1 mM catechol for 0 or 15 minutes at 37 °C before the centrifugation. Two independent experiments were done for each incubation. Therefore, whole cells, nuclei, cytoskeletons and plasma membranes were removed by centrifugation at 550 ***g*** for 10 minutes at 4 °C, followed by centrifugation of the supernatant at 7,100 ***g*** for 10 minutes at 4 °C. The mitochondrial pellet was resuspended in the same buffer and washed twice at 6,400 **g** for 10 minutes at 4 °C. Finally, mitochondria were resuspended in 1 ml of the same isolation buffer. Protein determinations were performed as described for nuclear fractions.

Oxygen electrode measurements

Oxygen consumption was carried out at 37 °C in a closed chamber containing a Clark type oxygen electrode connected to a YSI model 53 monitor (Yellow Springs Instrument Co. Inc., OH, USA). Isolated mitochondria were suspended in 3 ml of 10 mM KCl, 0.2 mM EDTA, 0.25 M mannitol, 0.025% (w/v) BSA, 10 mM Tris, 5 mM phosphate buffer, pH 7.4 at a final concentration of 0.4 mg protein/ml. Sodium succinate was added to a final concentration of 10 mM in order to induce mitochondrial basal respiration (state 2). Oxygen uptake in resting conditions was monitored for 12 minutes. Four assays were analyzed for each mitochondrial fraction.

RESULTS

Catechol inhibits mitochondrial basal respiration

The addition of catechol (1 mM) to brain homogenates resulted in brain mitochondrial inhibition of state 2 $FADH_2$-linked respiration **(Fig. 1)**. Catechol decreased O_2 uptake by 26% when mitochondrial fractions were obtained immediately after the addition of this compound to brain homogenates (P 0 min). Mitochondrial basal respiration was inhibited by 44% when 1 mM catechol was incubated for 15 minutes with brain homogenates prior to isolation of

mitochondria (P 15 min). Presumably catechol inhibits electron flow between Complex II and Complex IV through pathways mediated by phenolic or quinone group substituents on the phenyl ring.

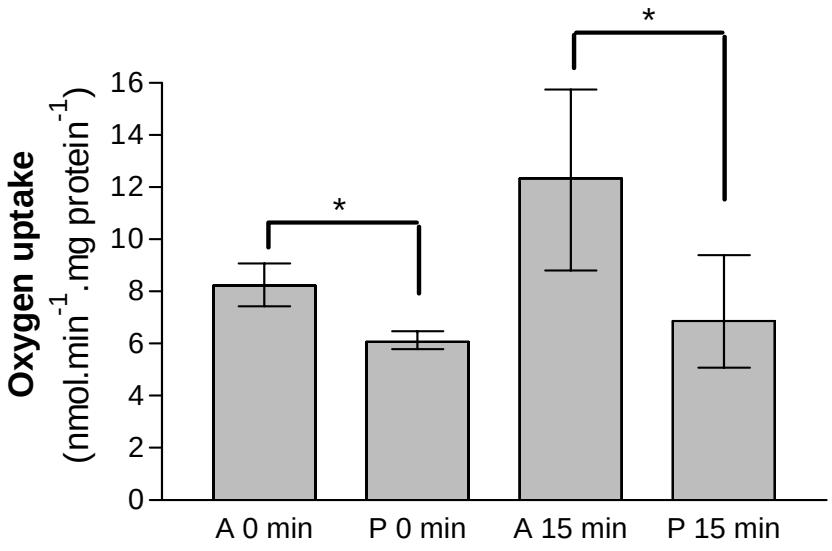

Fig. 1. Inhibition of mitochondrial basal respiration by catechol. Rat brain homogenates were incubated in the absence (A) or in the presence (P) of 1 mM catechol for 0 or 15 minutes prior to centrifugation. Two independent experiments were done for each incubation. Mitochondrial fractions were isolated as described in Materials and Methods. Mitochondria (0.4 mg protein) were incubated at 37 °C in 10 mM KCl, 0.2 mM EDTA, 0.25 M mannitol, 0.025% (w/v) BSA, 10 mM Tris, 5 mM phosphate buffer, pH 7.4. Mitochondrial basal respiration was measured for 12 minutes using a Clark O_2 electrode after the injection of 10 mM sodium succinate. Four assays were carried out for each mitochondrial fraction (n = 8 per group). Results are expressed as median ± 25^{th} and 75^{th} percentiles. Statistical significance was analyzed using Mann-Whitney U test. *, $P < 0.05$.

DISCUSSION

We showed in this work that catechol is capable of inhibiting brain mitochondrial state 2 $FADH_2$-linked respiration. These data suggest inhibition of mitochondrial respiration by catechol as a potential mechanism of its cytotoxicity. Other laboratories have shown that endogenous catechols and exogenous molecules bearing a catechol moiety are also inhibitors of mitochondrial respiration. Endogenous cysteinylcatechols are potent inhibitors of mitochondrial complex I activity in vitro [6]. Dopamine and its metabolite 3,4-dihydroxyphenylacetic acid can inhibit brain mitochondrial state 3 NADH-linked

respiration [7]. Flavonoids with a catechol on their rings were inhibitors of state 2 $FADH_2$- and NADH-linked respiration [8]. A previous study about catechol-O-methyltransferase inhibitors demonstrated the effects of 3,4-dihydroxy-4'-methyl-5-nitrobenzophenone (tolcapone) in decreasing respiratory control ratio in mitochondrial preparations at low micromolar concentrations [9]. Furthermore, tolcapone reduced ATP synthesis in human neuroblastoma SH-SY5Y cells [10].

CONCLUSION

In this work we demonstrated that catechol induced a partial inhibition of state 2 $FADH_2$-linked mitochondrial respiration, probably due to oxidation of this molecule and consequently reactive oxygen species formation.

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4 COMENTÁRIOS, CRÍTICAS E CONCLUSÕES

O trabalho desenvolvido para a elaboração desta dissertação permitiu avaliar os aspectos toxicólogicos de compostos derivados do benzeno e tolueno em um modelo mitocondrial.

O protocolo experimental permitiu elucidar que os catecóis (pirocatecol e 3-metilcatecol) têm a habilidade de induzir uma inibição parcial da respiração basal mitocondrial ligada ao $FADH_2$ (utilizando-se succinato como substrato – Complexo II). No entanto, experimentos adicionais, tais como a utilização de ADP, têm sido cogitado para assegurar um maior entendimento sobre o efeito de catecóis na cadeia respiratória.

Paralelamente, testamos a hipótese de catecóis (pirocatecol) produzirem dano ao DNA em células cerebrais de ratos. Observamos que a injúria é de forma dose-dependente, constatando-se que o catecol mesmo em pequenas concentrações (1mM) é capaz de produzir um significativo dano ao material genético celular. No entanto, estudos adicionais são importantes e necessários, no que tange à utilização de uma enzima reparadora (FPG) e um modelo antioxidativo.

Sumariamente, podem ser postulados os seguintes pontos como conclusão geral:

1. Catecóis derivados do Benzeno (pirocatecol) e Tolueno (3-metilcatecol) foram capazes de inibir a respiração basal associada ao $FADH_2$ em mitocôndrias isoladas de fígado e cérebro de ratos, o que pode levar à toxicidade, produção de espécies reativas e morte celular.
2. Estudos adicionais demonstraram que o pirocatecol induziu um dano oxidativo de DNA, expresso com o aumento na ocorrência de cometas de Níveis 2 a 4 de forma dose dependente, sugerindo que as espécies reativas de oxigênio geradas pela oxidação do catecol induzem danos de DNA, o que pode também estar relacionado com a citotoxicidade deste composto.

Finalizando, é importante ressaltar que os estudos aqui apresentados são conclusivos no que diz respeito aos efeitos tóxicos de catecóis (pirocatecol e 3-metilcatecol) na respiração basal mitocondrial ligada ao $FADH_2$ (Complexo II). Porém, ainda é necessária a utilização de outros substratos para o estudo deste efeito nos demais complexos mitocondriais. No modelo celular de cérebro de ratos, o pirocatecol demonstrou induzir um dano oxidativo de DNA importante, no entanto, estudos adicionais são importantes no intuito de elucidar melhor o comportamento desse composto quando confrontado com moléculas antioxidantes e/ou uma enzima reparadora.

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Abstract

Aim: The aim of this work was to investigate the hypothesis that catechol and 3MC inhibit $FADH_2$-linked basal respiration in mitochondria isolated from rat liver and brain homogenates. Moreover, catechol ability to induce DNA damage in rat brain cells through the comet assay (alkaline single-cell gel electrophoresis assay) was also observed. **Methods:** Two different catechols were evaluated: pirocatechol (derived from benzene) and 3-methylcatechol (derived from toluene); rat liver and brain homogenates were incubated with 1mM catechol at pH 7.4 for up to 30 minutes. After that, mitochondrial fractions were isolated by differential centrifugation. Basal oxygen uptake was measured using a Clark-type electrode after the addition of 10 mM sodium succinate for a period of 12 minutes. In additional experiments, rat brain cells were treated with 1, 5 and 10mM pirocatechol for up to 20 minutes at 37º C, and submitted to electrophoresis. **Results:** Catechols (pirocatechol and 3-methylcatechol) induced a time-dependent partial inhibition of $FADH_2$-linked basal mitochondrial respiration. Indeed, pirocatechol was able to produce a dose-dependent DNA oxidative damage in rat brain cells by 2 and 4 injury levels. These results suggest that reactive oxygen species generated by the oxidation of catechols, induced an impairment on mitochondrial respiration and a DNA damage, which might be related to their citotoxicity. **Conclusion:** Catechols produced an inhibition of basal respiration associated to $FADH_2$ in isolated liver and brain mitochondria; 3-methylcatechol, at the same concentration, produced similar toxicity in the mitochondrial model. Indeed, pirocatechol induced a DNA damage in rat brain cells, mainly observed in comets formation and consequent DNA degradation.

Key Words: benzene, toluene, catechols, free radicals, respiration.

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